Keratins and the Keratinocyte Activation Cycle

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In wound healing and many pathologic conditions, keratinocytes become activated: they turn into migratory, hyperproliferative cells that produce and secrete extracellular matrix components and signaling polypeptides. At the same time, their cytoskeleton is also altered by the production of specific keratin proteins. These changes are orchestrated by growth factors, chemokines, and cytokines produced by keratinocytes and other cutaneous cell types. The responding intracellular signaling pathways activate transcription factors that regulate expression of keratin genes. Analysis of these processes led us to propose the existence of a keratinocyte activation cycle, in which the cells first become activated by the release of IL-1. Subsequently, they maintain the activated state by autocrine production of proinflammatory and proliferative signals. Keratins K6 and K16 are markers of the active state. Signals from the lymphocytes, in the form of Interferon-γ, induce the expression of K17 and make keratinocytes contractile. This enables the keratinocytes to shrink the provisional fibronectin-rich basement membrane. Signals from the fibroblasts, in the form of TGF-β, induce the expression of K5 and K14, revert the keratinocytes to the healthy basal phenotype, and thus complete the activation cycle. ***J Invest Dermatol 116:633–640, 2001***

Epidermal keratinocytes have two alternative pathways open to them: differentiation and activation. In healthy epidermis, keratinocytes differentiate from the basal layer through squamous, granular, and cornified layers. This process has been described in several review articles recently (Eckert *et al*, 1997; Fuchs *et al*, 1997; Mischke, 1998; Tomic-Canic *et al*, 1998). From the perspective of this paper, we point out that the differentiation process can be affected by vitamins, such as retinoic acid and vitamin D3, and that the expressions of specific keratin genes have been often used as markers for basal *versus* differentiating cells: K5 and K14 are expressed in the basal layer, K1, K2, and K10 in the differentiating cells (reviewed in Schweizer, 1993). In response to epidermal injury, however, or in certain pathologic conditions such as psoriasis, an alternative pathway is open to keratinocytes, that of activation (reviewed in Barker *et al*, 1991; Nickoloff and Turka, 1993; Kupper and Groves, 1995; Tomic-Canic *et al*, 1998; Murphy *et al*, 2000). The activation process can be affected by growth factors and cytokines, such as interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), transforming growth factor α (TGF-α), TGF-β, and interferon-γ (IFN-γ). The expression of specific keratin genes has been used as a marker for activated cells; characteristically, activated keratinocytes express K6, K16, and K17 keratin proteins, distinct from the keratins of the healthy epidermis. Activated keratinocytes are hyperproliferative, migratory, change their cytoskeleton, augment the levels of cell surface receptors, and produce components of the basement membrane. These responses are essential for re-epithelialization of the injured area. Activated keratinocytes also produce paracrine signals to alert fibroblasts, endothelial cells, melanocytes, and lymphocytes, as well as autocrine signals targeted at neighboring keratinocytes. These responses are essential for orchestrating the actions of the surrounding cell types in repair of the injured tissue. The affected cell types, in turn, produce their own autocrine and paracrine signals, which modify the actions of activated keratinocytes. Eventually, having responded to the injury, keratinocytes receive a "de-activation" signal and revert to the normal differentiation pathway. The regulatory processes involved in keratinocyte activation and de-activation, as well as the concomitant changes in keratin gene expression, are coordinated by secreted growth factors and cytokines, produced both by the keratinocytes and by the surrounding cell types. These regulatory processes are the subject of this review.

INITIATOR OF ACTIVATION: IL-1

In healthy epidermis, keratinocytes are not activated and they slowly proliferate in the basal layer and differentiate in the suprabasal layers. Being exposed to the surroundings, however, they must be prepared to respond very quickly to injury from the environment. Therefore, keratinocytes produce sentinel molecules ready to signal promptly that an injury has occurred and the tissue needs to become activated. Activated keratinocytes repair the tissue and eventually become deactivated, reverting to normal differentiation. This process, termed the keratinocyte activation cycle, is

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Abbreviations: ERK, extracellularly regulated kinase; IKK, IkB kinase; IRAK, IL-1 receptor associated kinase; JAK, Janus activated kinase; MAPK, mitogen activated protein kinase; MEK, MAPK/ERK kinase; NIK, NFkB inducing kinase; PKC, protein kinase-C; TAK, TRAF associated kinase; TRADD, TNFα receptor associated death domain; TRAF, TNFα receptor associated factor.

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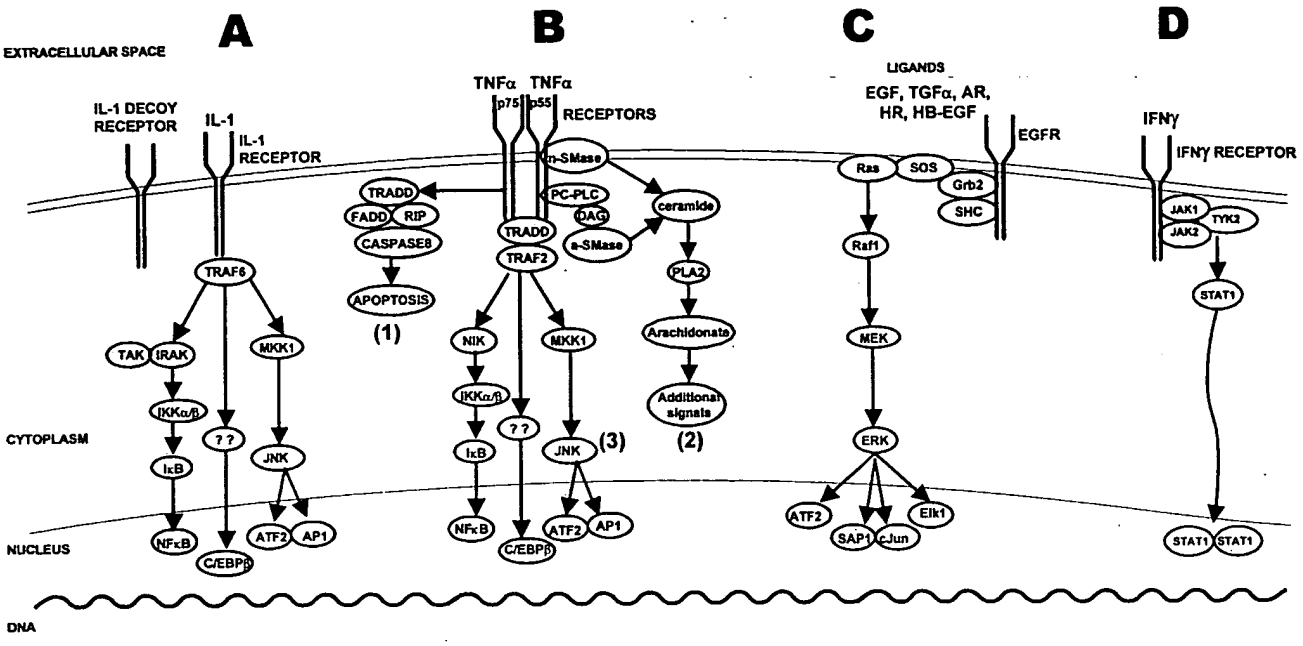

Figure 1. Signaling pathways in keratinocytes. (*A*) The IL-1 signal transduction pathways. The receptor interacts with TRAF6, which causes activation of protein kinases TAK, IRAK, and MKK1. This results in activation of transcription factors, such as NFκB, C/EBPβ, ATF2, and AP-1. (*B*) The TNF-α signal transduction pathways. There are three principal signal transduction pathways: (1) the apoptosis pathway; (2) the ceramide pathway; and (3) the TRAF2 pathway. The apoptosis pathway proceeds through a "death domain" containing proteins TRADD and FADD. In the ceramide pathway, PC-PLC stand for phosphatidyl-choline-activated phospholipase-C, DAG for diacyl-glycerol, n-SMase and a-SMase for neutral and acidic sphingomyelinase, and PLA2 for phospholipase-A2. TRAF2, *via* kinases NIK and IKKs, phosphorylates and causes subsequent degradation of IκB, which allows NFκB to become activated and enter the nucleus. TRAF2 also activates the MKK1 and JNK pathways. The mechanisms activating C/EBPβ have not yet been elucidated. (*C*) The TGF-α signal transduction pathways. Growth factors, such as TGF-α, EGF, etc., bind to EGFR activating the cytoplasmic tyrosine kinase. Activated kinase binds scaffolding proteins, such as SHC, Grb2, and SOS, bringing them in the close proximity of Ras. They activate Ras, which activates Raf1, which activates MEKs, which activate ERKs. When activated, ERKs translocate to the nucleus, where they phosphorylate and thus activate transcription factors, such as ATF2, SAP1, c-Jun, and Elk1. (*D*) The IFN-γ signal transduction pathway. Binding of the ligand to the receptor causes its association with the JAK/TYK kinases, which phosporylate STATs. STATs, when phosphorylated, dimerize and translocate to the nucleus where they activate transcription.

governed by extracellular signals, and is characterized by changes in expression of keratin proteins.

The most common initiator of keratinocyte activation is IL-1. Both the α and the β form of this cytokine are present unprocessed in the cytoplasm of keratinocytes. They are unavailable for binding to the cell surface receptors because they are sequestered in the cytoplasm (Hauser *et al*, 1986; Kupper *et al*, 1986a; Mizutani *et al*, 1991a; Kupper and Groves, 1995). Cytoplasmic IL-1 stands sentry in the epidermis, ready to respond to injury. Injured keratinocytes process and release IL-1, allowing the surrounding cells to perceive it (Kupper *et al*, 1986b; Murphy *et al*, 1989; Bochner *et al*, 1990; Mizutani *et al*, 1991b; Chan *et al*, 1992; Wood *et al*, 1996; Yu *et al*, 1996; Lundqvist and Egelrud, 1997; Zepter *et al*, 1997; Corsini *et al*, 1998; Murphy *et al*, 2000). The released IL-1 serves as a paracrine signal to dermal endothelial cells to become activated, express selectins, and slow down the circulating lymphocytes (Cartwright *et al*, 1995; Lee *et al*, 1997; Romero *et al*, 1997; Wyble *et al*, 1997). IL-1 also serves as a chemoattractant for lymphocytes, causing them to extravasate and migrate to the site of injury (Nourshargh *et al*, 1995; Santamaria Babi *et al*, 1995). Furthermore, IL-1 is an activator of dermal fibroblasts, enhancing their migration, proliferation, and production of dermal extracellular matrix components (Mauviel *et al*, 1991; 1993; Godessart *et al*, 1994; Maas-Szabowski and Fusenig, 1996). IL-1 is also an autocrine signal that activates keratinocytes. IL-1 causes them to proliferate, become migratory, and express an activation-specific set of genes (Kupper, 1990a; Gyulai *et al*, 1994; Chen *et al*, 1995; Tomic-Canic *et al*, 1998).

Keratinocytes express IL-1 receptors, both the type I, functional, and the type II, decoy, on their surface, as well as the IL-1 receptor antagonist (Blanton *et al*, 1989; Stosic-Grujicic and Lukic, 1992; Kutsch *et al*, 1993; Eller *et al*, 1995; Grewe *et al*, 1996; Debets *et al*, 1997; Rauschmayr *et al*, 1997). The epidermal responses to IL-1 are exquisitely finely tuned: keratinocytes must be ready to respond quickly to injury via IL-1 and at the same time must be able to attenuate and shut off the IL-1 signals after the initial response.

Signal transduction in response to IL-1 starts at the cell surface with the type I receptor. The intracellular domain of this receptor associates with several proteins, e.g., TNFα receptor associated factor (TRAF)-6, which recruit protein kinases such as IL-1 receptor associated factor (IRAK) and TRAF associated kinase (TAK). Downstream from the kinases, the signal trifurcates and at least three transcription factor systems are activated: the NFκB, C/EBPβ, and AP-1 (**Fig 1*A***) (Cao *et al*, 1996; Muzio *et al*, 1997; La and Greene, 1998; Baud *et al*, 1999; Lomaga *et al*, 1999; Ninomiya-Tsuji *et al*, 1999; Ling and Goeddel, 2000). These transcription factors then induce expression of the activation-specific proteins.

Among genes induced by IL-1 are growth factors and cytokines that transmit the signals of the specific type of injury to the surrounding cells. These include granulocyte-macrophage colony stimulating factor (GM-CSF), TNF-α, TGF-α, amphiregulin, additional IL-1, etc. (Kupper *et al*, 1988; Larsen *et al*, 1989; Tosato and Jones, 1990; Lyons *et al*, 1993; Lee *et al*, 1994; Chen *et al*, 1995; Lontz *et al*, 1995; Bechtel *et al*, 1996; Chung *et al*, 1996; Fujisawa *et al*, 1997a, b; Nylander-Lundqvist and Egelrud, 1997; Kozlowska *et al*, 1998). Activated keratinocytes also produce cell surface markers, such as intercellular adhesion molecule 1 (ICAM-1) and integrins as well as fibronectin, a component of the basement membrane that promotes keratinocyte migration (Kubo *et al*, 1984; O'Keefe *et al*, 1987; Griffiths *et al*, 1989; Lisby *et al*, 1989; Clark, 1990; Guo *et al*, 1991; Grinnell, 1992; Krutmann *et al*, 1992; Middleton and Norris, 1995).

Among the genes induced by IL-1 are keratins K6 and K16. Whereas the mechanism of induction of K16 is still under investigation, many details of the induction of K6 are known. Recently, we reported on the mechanism of induction of K6 by IL-1 (Komine *et al*, 2001). Skin biopsies in organ culture treated with IL-1 express K6 throughout the tissue. In cultures only confluent keratinocytes respond to IL-1; subconfluent cultures do not. Using DNA-mediated cell transfection, we identified the IL-1 responsive DNA element in the K6 promoter, and determined that it contains a complex of C/EBP binding sites. Thus, IL-1 initiates keratinocyte activation not only by triggering additional signaling events, but also by inducing directly the synthesis of K6 in epidermal keratinocytes, and thus changing the composition of their cytoskeleton.

MAINTENANCE OF ACTIVATION

Whereas IL-1 initiates the keratinocyte activation, other signals are used to maintain keratinocyte activation. Such signals need not be already present in healthy tissue and can have overlapping but different mechanisms of action from IL-1. Because these signals are not present in healthy tissue, keratinocytes do not need to elaborate sophisticated hair-trigger mechanisms to respond to or protect themselves from these signals. One such signal is TNF-α. Induced by IL-1, TNF-α can maintain keratinocytes in an activated state (Nickoloff and Turka, 1993).

TNF-α was discovered from two independent lines of research, first as an inducer of necrosis in some tumor cells and second as a cause of cachexia in septic animals. Subsequently, it was established that TNF-α is one of the proinflammatory cytokines that induce many inflammatory effects, such as fever and shock. In response to infection or injury a wide variety of cells produce TNF-α, primarily macrophages and monocytes but also epithelial cells including keratinocytes (Kock *et al*, 1990; Nickoloff *et al*, 1991; Kolde *et al*, 1992).

A low level of TNF-α is present in the upper layers of the healthy epidermis, but IL-1 can induce its synthesis and release from keratinocytes. The levels of TNF-α are greatly augmented under a variety of conditions, such as allergic and irritant contact dermatitis, infection, and ultraviolet irradiation (Barker *et al*, 1991). In these pathologic conditions TNF-α activates immune responses by inducing production of additional signaling molecules, cytokines, growth factors, their receptors, and adhesion proteins (e.g., amphiregulin, TGF-α, IL-1α, IL-1 receptor antagonist, epidermal growth factor receptor (EGFR), and ICAM-1 (Griffiths *et al*, 1995, and references therein).

The signaling cascades mediating cellular responses to TNF-α have been partly elucidated (Rothe *et al*, 1994; 1995; Liu *et al*, 1996; Shu *et al*, 1996; Malinin *et al*, 1997; Natoli *et al*, 1997; Regnier *et al*, 1997; Song *et al*, 1997). The effects of TNF-α partly overlap those of IL-1, but the TNF-α-dependent signal transduction appears to be much more complicated than the IL-1-triggered one (although it is possible that at the moment we see too many trees, which perhaps obscures the forest). A current version of the cascade is shown in **Fig 1(*B*)**. There are two TNF-α receptors, but keratinocytes express mainly the 55 kDa receptor, type 1 (Trefzer *et al*, 1991; Kristensen *et al*, 1993; Kondo and Sauder, 1997). Three major intracellular effects are caused by TNF-α. The first is the induction of apoptosis, which proceeds through activation of caspases. The second involves production of ceramides, which in turn act as second messengers activating arachidonic acid synthesis and regulating downstream effects. Ceramides activate protein kinases that feed into the mitogen activated protein kinase (MAPK) cascade system. The third and most direct TNF-α signaling pathway involves proteins TNFα receptor associated death domain (TRADD) and TRAF2, which, through NFκB inducing kinase (NIK) and other kinases, activate transcription factors NFκB and C/EBPβ. The same pathway activates members of the AP-1 transcription factor family. There is significant crosstalk between the TNF-α signaling and the MAPK cascade pathways.

The NFκB family includes the proteins p65, p50, and c/rel, which both homodimerize and heterodimerize among themselves (Miyamoto and Verma, 1995). These proteins are stored latent in the cytoplasm, bound to the inhibitory protein IκB. TNF-α causes activation of IKKs, kinases that phosphorylate IκB and induce its degradation. The degradation of IκB results in activation and nuclear translocation of the NFκB protein (Beg *et al*, 1993; Shu *et al*, 1996; Regnier *et al*, 1997; Zandi *et al*, 1998). Knockout of IKK-α has a severe epidermal phenotype causing incomplete epidermal differentiation (Hu *et al*, 1999; Takeda *et al*, 1999). On the other hand, a knockout of IKK-β is defective in signaling from TNF-α to NFκB (Li *et al*, 1999a; 1999b). NFκB proteins can interact with C/EBPβ, AP-1, and other transcription factors to regulate gene expression (Matsusaka *et al*, 1993; Stein *et al*, 1993). In keratinocytes, *in vitro* overexpression of NFκB inhibits proliferation. In epidermis *in vivo* NFκB is present in all layers, but is nuclear only in the suprabasal ones; this suggests a role for NFκB in epidermal differentiation (Seitz *et al*, 1998). On the other hand, constitutive activation of NFκB in IκB-knockout mice results in normal epidermal development and differentiation, but a widespread and lethal dermatitis in the first few days of life (Klement *et al*, 1996).

TNF-α and other extracellular stimuli activate transcription factor C/EBPβ (also known as NF-IL6 or LAP; Nakajima *et al*, 1993; Trautwein *et al*, 1993; Akira *et al*, 1997). The mechanisms that activate C/EBPβ have not been fully characterized. C/EBPβ interacts with many other transcription factors, such as the RB protein, the glucocorticoid receptor, Myc, NFκB, and AP-1 (Brasier *et al*, 1990; Matsusaka *et al*, 1993; Nishio *et al*, 1993; Stein and Baldwin, 1993; Klampfer *et al*, 1994; Chen *et al*, 1996; Mink *et al*, 1996). In epidermis the C/EBP proteins are differentially expressed during differentiation (Maytin and Habener, 1998; Oh and Smart, 1998). Whereas knockout mice lacking C/EBPβ have no cutaneous phenotype (Tanaka *et al*, 1995), overexpression of C/EBPβ in keratinocytes causes growth arrest and induction of early differentiation markers (Zhu *et al*, 1999).

Using cultured keratinocytes and a novel *ex vivo* system, we showed that TNF-α induces the expression of K6 at the level of transcription (Komine *et al*, 2000). Using cotransfection, specific inhibitors, and antisense oligonucleotides, we have identified NFκB and C/EBPβ as the transcription factors that convey the TNF-α signal. Both are necessary for the induction and they apparently act as a complex, although only C/EBPβ binds the K6 promoter DNA. The site in the K6 gene promoter that responds to TNF-α is separate from the site responsive to TGF-α. These results show that the inflammatory (TNF-α) and the proliferative (TGF-α) signals in epidermis regulate the expression of K6 separately and independently. Thus the cytoskeletal responses, such as K6 synthesis, can be precisely tuned in epidermal cells by separate proinflammatory and proliferative signals to fit the nature of the injuries that caused them.

Whereas IL-1 and TNF-α are proinflammatory signals with overlapping intracellular molecular pathways, under certain conditions keratinocytes need additional and different stimuli, which direct them to proliferate. In epidermis, several members of the EGF family can be produced, including TGF-α, amphiregulin, HB-EGF, and heregulin, ligands of the EGFR. These convey proliferative signals to keratinocytes.

Arguably the most extensively studied cellular receptor signaling pathways are those proceeding through EGFR (Ullrich and Schlessinger, 1990). In adult epidermis, EGFR is primarily expressed in the basal layer and, to a lesser degree, the first suprabasal layers (Nanney *et al*, 1990). Binding of the appropriate ligands to the EGFR can activate keratinocytes (Coffey *et al*, 1987). The signals activate nuclear proteins that regulate both gene expression and cell division. Among the regulated genes are those encoding additional regulators, leading to major morphologic changes, developmental changes, and differentiation. In response to the activation of the EGFR, keratinocytes proliferate, degrade

components of the extracellular matrix, and become migratory (Nickoloff *et al*, 1990).

A "simplified" scheme of the cascade is shown in **Fig 1(*C*)**. The binding of a ligand to EGFR causes the receptor to dimerize, with concomitant activation of its intracellular protein tyrosine kinase. A substrate for this kinase is the receptor itself – the two monomers phosphorylate each other. The phosphotyrosines serve as docking sites for SH2 domain containing proteins (such as Grb2 or SHC) that interact with proteins capable of activating Ras. Several growth factor receptors, *via* different adaptor molecules, activate Ras, which makes Ras a fulcrum for signal transduction pathways (**Fig 1*C***). Activated Ras, in turn, activates a cascade of three protein kinases, Raf1, MAPK/ERK kinase (MEK), and extracellularly regulated kinase (ERK). The last one, ERK, translocates to the nucleus where it phosphorylates and thus activates transcription factors such as Elk1 and SAP1 (reviewed in Ullrich and Schlessinger, 1990; Hill and Treisman, 1995).

Successive activation of a cascade of three protein kinases, first characterized in the EGF/TGF-α signaling pathway, is a recurrent motif in signal transduction. Stress, exemplified by osmotic shock and ultraviolet irradiation, or proinflammatory cytokines including TNF-α and IL-1, can activate parallel cascades (see above), thus activating partially overlapping sets of transcription factors (Dérijard *et al*, 1994; Galcheva-Gargova *et al*, 1994; Gupta *et al*, 1995; 1996; Rosette and Karin, 1995). All these cascades are present and functional in keratinocytes (M.B. unpublished).

Perhaps the best-characterized TGF-α-responsive transcription factors are those belonging to the AP-1 family. AP-1 is a nuclear transcription complex composed of dimers encoded by the *fos* and *jun* families of proto-oncogenes (Hill and Treisman, 1995; Karin, 1996). Whereas Fos proteins only heterodimerize with members of the Jun family, Jun proteins can dimerize with both Fos and other Jun proteins. In the epidermis, AP-1 regulates cell growth, differentiation, and transformation (Bernerd *et al*, 1993; Saez *et al*, 1995; Rutberg *et al*, 1996). The expression of individual AP-1 proteins in epidermal layers, however, is a controversial issue that awaits resolution. Certain authors find c-Fos in lower layers of the epidermis. (Fisher *et al*, 1991; Basset-Seguin *et al*, 1994; Lu *et al*, 1994) whereas others do not find any c-Fos (Rutberg *et al*, 1996), which agrees with the lack of an epidermal phenotype in c-*fos* knockout mice (Saez *et al*, 1995). The differing results could be explained by different epitopes of the antibodies used and functional redundancy of Fos family members. Be that as it may, it is clear that the AP-1 proteins in keratinocytes can regulate the expression of differentiation markers (Presland *et al*, 1992; Lu *et al*, 1994; Lohman *et al*, 1997) and may convey the calcium- and protein kinase C (PKC) dependent signals (Welter *et al*, 1995; Rutberg *et al*, 1996). Functional AP-1 sites have been found in many keratin genes, including the first intron of human and murine K18 and the K8 gene (Pankov *et al*, 1994; Umezawa *et al*, 1997). We have found that the EGFR ligands strongly and specifically induce the expression of K6 and K16 and that AP-1 sites are present and functional in several epidermal keratin genes (Jiang *et al*, 1993; Ma *et al*, 1997)

THE ACTIVATED PHENOTYPE

Once activated, keratinocytes synthesize additional signaling growth factors and cytokines including TGF-α, IL-3, IL-6, IL-8, G-CSF, GM-CSF, and M-CSF (Coffey *et al*, 1987; Kupper, 1990b; Nickoloff *et al*, 1990). The effects of these signaling molecules produced by keratinocytes are chemotactic for white blood cells and paracrine for lymphocytes, fibroblasts, and endothelial cells. Interestingly, these signaling molecules are also autocrine for keratinocytes themselves. They lead to secondary effects of keratinocyte activation. Several extracellular markers are specifically expressed by the activated keratinocytes. These include cell surface proteins, integrins, components of the extracellular matrix, as well as receptors for both the autocrine factors and factors produced by the infiltrating immune cells (Alitalo *et al*, 1982; O'Keefe *et al*, 1987; Marinkovich *et al*, 1992; Burgeson, 1993). In a feedback loop, the increase in the expression of cell surface receptors may augment the initial activation signal. The various signaling molecules may be synergistic or antagonistic with each other. This allows the activated phenotype to be specifically modified, which can lead to different activated phenotypes. Put simply, keratinocytes activated during wound healing, in psoriasis, or other pathologic conditions can have different variants of the activated keratinocyte phenotype.

THE CONTRACTILE KERATINOCYTE: IFN-γ

In the late stages of wound healing, the contraction of the newly formed extracellular matrix produced by the fibroblasts is an important process. This contraction is effected by fibroblasts; however, keratinocytes have their own task, to contract the newly deposited, fibronectin-rich basement membrane. The signal that compels keratinocytes to become competent to contract is, apparently, IFN-γ.

The most extensively studied signaling molecules of the immune system are the interferons IFN-α, IFN-β, and IFN-γ, a subset of cytokines originally described as factors that protect cells from viral infections (reviewed in Schindler and Darnell, 1995). IFN-α and IFN-β share a cell surface receptor, whereas IFN-γ binds to a different receptor and has distinct effects. Certain diseases, such as psoriasis, are associated with high levels of IFN-γ in epidermis (Nickoloff *et al*, 1990). Although the role of interferons in pathologic processes has not been clearly defined, they have been used in therapeutic trials for several dermatologic diseases (Eron *et al*, 1987).

Activation of IFN receptors initiates a cascade of protein phosphorylation events. The cascade branches into a delta of transcription activating pathways that induce multiple genes (Schindler and Darnell, 1995). The receptors interact with Janus activated kinases (JAK) kinases, which phosphorylate tyrosines both on the receptors and on the signal transducing activator of transcription (STAT) family of transcription factors (**Fig 1*D***). First discovered as mediators of interferon signaling, STATs are unusual because they can convey the signal directly from the plasma membrane into the nucleus without second messengers or cytoplasmic kinase cascade intermediates (Levy and Darnell, 1990). Each STAT contains a tyrosine phosphorylation site and an SH2 domain that can bind to phosphotyrosine. STATs are cytoplasmic in their ground state, but upon activation of appropriate receptors they become phosphorylated and, through their SH2 domains, dimerize and translocate into the nucleus. In the nucleus STATs bind to specific DNA recognition elements and activate transcription of nearby genes. To date six STAT proteins have been characterized; they are activated by a variety of extracellular stimuli. The regulatory specificity of the cytokine signals at the cell surface is mirrored in the nucleus by the activity of specific members of the STAT family: IFN-γ leads to activation of STAT-1, IFN-α of STAT-2 and STAT-3, IL-6 and OsM of STAT-3, IL-12 of STAT-3 and STAT-4, IL-3, IL-5, and GM-CSF of STAT-5, and IL-4 of STAT-6 (Schindler and Darnell, 1995).

We found that IFN-γ strongly and specifically induced the promoter of the K17 gene. No other keratin gene construct was induced (Jiang *et al*, 1994). Within the promoter of the K17 gene, we have identified and characterized a site that confers the responsiveness to IFN-γ, and that binds the transcription factor STAT-1. We could induce *in vivo* expression of K17 experimentally by causing a delayed-type hypersensitivity inflammatory reaction characterized by substantial infiltration of lymphocytes that produce IFN-γ (Kaplan *et al*, 1986). In affected epidermis, we found transcription factor STAT-1 in the nuclei of keratinocytes. In contrast, STAT-1 is cytoplasmic in unaffected and healthy skin.

Psoriasis is a Th-1-dependent process that is associated with production of IFN-γ. We hypothesized that the induction of K17 is specific for Th-1 inflammatory reactions and does not occur in Th-2 type ones. Therefore, we analyzed lesional samples of psoriasis

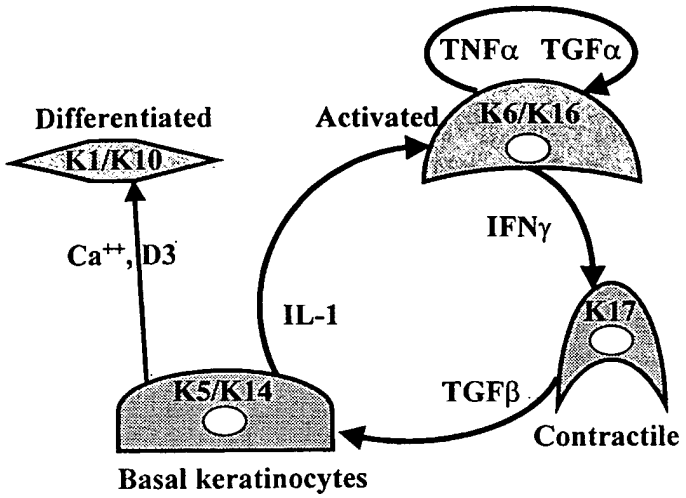

Figure 2. The keratinocyte activation cycle. Basal keratinocytes, producing K5 and K14, can either differentiate and produce K1 and K10, or become activated, producing K6 and K16. IL-1 is the primary signal initiating keratinocyte activation and expression of K6 and K16. TNF-α and TGF-α keep keratinocytes activated until another signal, such as IFN-γ, is received. IFN-γ induces K17 and promotes contractility in keratinocytes. TGF-β is a de-activating signal that promotes reversal to the basal phenotype and induces expression of K5 and K14.

and compared them with those of atopic dermatitis, a Th-2-associated process. The above hypothesis has been supported by our evidence that K17 is induced in the first, but not in the second, disorder (Komine *et al*, 1996). Our data further indicated that Th-1 and Th-2 lymphocytes, through the cytokines they produce, differently regulate not only each other, but also keratin gene expression in epidermis, their target tissue (Komine *et al*, 1996). These results characterize, at the molecular level, a signaling pathway produced by the infiltration of lymphocytes in skin and resulting in the specific alteration of gene expression in keratinocytes. They define at the molecular level how IFN-γ regulates expression of the K17 gene and provide a means for analysis of the molecular interactions between the immune system and the epidermis, interactions that are important in pathologic skin processes (Komine *et al*, 1996).

K17 is exceptional because it is not found in healthy interfollicular epidermis, but it is expressed in certain pathologic states, including psoriasis, allergic reactions, and cutaneous T cell lymphoma, as well as in benign tumors of the mammary gland, basal cell epitheliomas, squamous cell lung carcinomas, and some other benign and malignant neoplasms (Moll *et al*, 1984; Guelstein *et al*, 1988; de Jong *et al*, 1991; Wetzels *et al*, 1991; Blumenberg, 1994; Jiang *et al*, 1994). Indeed, expression of K17 has been used to evaluate the course of treatment of psoriatic patients (de Jong *et al*, 1991).

K17 is expressed in various healthy epithelia (Troyanovsky *et al*, 1992), including myoepithelial cells, basal layers of transitional and pseudostratified epithelia of the respiratory and urinary tracts, and early developmental stages of stratified epithelia. Common characteristics of these cells are contractility and/or frequent changes in shape (Troyanovsky *et al*, 1992). The function of K17 in epidermis therefore may be to promote or allow keratinocyte contractility.

BACK TO BASICS: TGF-β

Once the injury that causes keratinocyte activation has been healed and the tissue repaired, keratinocytes must revert to their regular function, differentiation into stratum corneum. To revert to the basal cell phenotype, keratinocytes need a signal that the injury is over. This signal comes from the dermal fibroblasts in the form of TGF-β.

TGF-β is an important regulator of epidermal keratinocyte function because it suppresses cell proliferation, whereas it induces synthesis of extracellular matrix proteins and their cell surface receptors. Mice with a knocked-out TGF-β gene develop normally, because of the maternally supplied TGF-β, only to succumb to exuberant multifocal inflammation due to unrestrained activation of the immune system (Shull *et al*, 1992; Geiser *et al*, 1993). Skin-targeted overexpression of TGF-β causes hypoplasia, whereas loss of TGF-β expression or resistance to TGF-β cause increased susceptibility to malignant conversion (Jhappan *et al*, 1993; Glick *et al*, 1993; Pierce *et al*, 1993; Reiss *et al*, 1993; Sellheyer *et al*, 1993).

In skin, TGF-β induces expression of extracellular matrix and basement membrane components, such as fibronectin, laminin, and collagen IV and VII (Wikner *et al*, 1988; Ryynänen *et al*, 1991; Vollberg *et al*, 1991; König and Bruckner-Tuderman, 1992), extracellular proteases and their inhibitors (Edwards *et al*, 1987; Laiho *et al*, 1987; Salo *et al*, 1991; Keski-Oja and Koli, 1992), as well as cell surface proteins including integrins α5, αv, β1, β4, and β5, and bullous pemphigoid antigens BPAG1 and BPAG2 (Vollberg *et al*, 1991; Gailit *et al*, 1994). We have shown that TGF-β specifically induces synthesis of basal-cell-specific K5 and K14 (Jiang *et al*, 1995).

Overall, it appears that TGF-β promotes the synthesis of basal-cell-specific proteins and therefore promotes the basal phenotype. This happens at the expense both of the activated, hyperproliferative phenotype and of the differentiating phenotype. Our conclusion is strengthened by studies showing that the keratinocyte growth arrest by TGF-β is reversible, does not result in terminal differentiation, and can be modulated by regulators of keratinocyte differentiation, such as retinoic acid or calcium (Choi and Fuchs, 1990; Matsumoto *et al*, 1990; Wang *et al*, 1992). Furthermore, van Ruissen *et al* (1994) have shown, by using careful cytometric measurements, that *in vitro* TGF-β reduces the fast growth rate of keratinocytes to the slow level of cell division observed in the normal, nonhyperproliferative basal layer of skin *in vivo*. From these data we suggest that the effects of TGF-β on keratinocytes are not antiproliferative, but antihyperproliferative.

OVERVIEW

When we put all these data together, we arrive at a consistent framework for the action of growth factors and cytokines in epidermal injury (**Fig 2**). The first signal from the injury is the release of IL-1. This release activates endothelial cells and fibroblasts and invites lymphocytes to the wound site. At the same time, IL-1 activates keratinocytes, making them hyperproliferative and migratory, causing them to deposit a provisional fibronectin-rich basement membrane, express K6 and K16, and produce additional growth factors and cytokines, including TNF-α and members of the EGF family. These growth factors and cytokines maintain the keratinocytes in the activated state. Meanwhile, lymphocytes extravasate and migrate to the wound site to fight any infection and produce IFN-γ. IFN-γ is an autocrine signal activating the lymphocytes, but it is also a paracrine signal to keratinocytes, communicating the following message: "the infection is being dealt with; if the re-epithelialization is complete, it is time to express K17, to contract and reorganize the provisional basement membrane". Meanwhile, fibroblasts migrate to the wound site, producing extracellular matrix, expressing TGF-β. TGF-β is an autocrine signal activating the fibroblasts, but it is also a paracrine signal to keratinocytes, communicating the following message: "the dermis is being repaired; it is now time to start producing K5 and K14, to return to being a basal cell and the process of normal differentiation".

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Cytokines and irritant contact dermatitis

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Abstract

Skin irritation is a complex phenomenon that involves resident epidermal cells, fibroblasts of dermis, and endothelial cells as well as invading leukocytes interacting with each other under the control of a network of cytokines and lipid mediators. Keratinocytes play an important role in the initiation and perpetuation of skin inflammatory reactions through the release of, and responses to cytokines. While resting keratinocytes produce some cytokines constitutively, a variety of environmental stimuli, such as tumor promoters, ultraviolet light and chemical agents, can induce epidermal keratinocytes to release inflammatory cytokines (IL-1, TNF-α), chemotactic cytokines (IL-8, IP-10), growth promoting cytokines (IL-6, IL-7, IL-15, GM-CSF, TGF-α) and cytokines regulating humoral vs. cellular immunity (IL-10, IL-12, IL-18). The role of cytokines in xenobiotics-induced skin irritation and the early molecular events that follow the treatment with irritant compounds will be discussed.

Keywords: Skin irritation; Cytokines; Keratinocytes

1. Introduction

Skin irritation is defined as a non-immunological local reversible inflammatory reaction, characterized by erythema and edema following a single or repeated application of chemical to the identical cutaneous site. *Acute* irritant contact dermatitis is characterized predominantly by inflammation, while *chronic* irritant contact dermatitis is characterized predominantly by hyperproliferation and transient hyperkeratosis. Irritant contact dermatitis is a multifactorial disease, the onset of which depends on both intrinsic and extrinsic factors. Among the former, age, genetic background, race, sex, site and history of dermatitis may all be important. Moreover, the effects of irritants are related to the chemical properties of the molecule itself, which influence skin absorption (molecular weight, polarity, state of ionization, vehicle), to the concentration applied, to the duration of exposure, etc. (Berardesca and Distante, 1994).

Depending on the country, dermatoses comprise from 20–70% of all occupational diseases. Irritation of the skin is important, and it is commonly thought to account for approximately 60–80% of the clinically recognized human contact dermatitis. Most of the remaining contact

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dermatits represents allergic contact dermatitis (Wahlberg, 1996).

Substances that are keratin solvents, dehydrating agents, oxidizing or reducing agents, among others may be irritants. Due to the heterogenicity of skin irritants, there is no reliable method for assessing irritancy based on chemical structure.

The biochemical mechanisms involved in skin irritation are complex and not fully understood. Different skin irritants can trigger different inflammatory processes. In addition to destroying tissue directly, chemicals can alter cell functions and/or trigger the release, formation, or activation of autocoids, such as histamine, 'arachidonic acid metabolities', kinins, complement, reactive oxygen species and cytokines. Fig. 1 lists a hypothetical sequence of events following application of a skin irritant.

Cytokines represent a heterogenous family of inducible glycoproteins, produced by various cell types, which mediate local interaction and distant communication between cellular elements of immune and inflammatory responses. Cytokines may act directly, as inducers and regulators of cell growth, division and differentiation, as stimulators of cell movement and migration and as controllers of cellular function and interaction via induced changes in the expression of adhesion molecules and receptors for cytokines.

The purpose of this article is to examine the role of cytokines in experimental contact dermatitis and to investigate the early intracellular events that precede cytokine induction following in vitro treatment of keratinocytes with irritant compounds.

2. Epidermal cytokines, keratinocytes and skin irritation

The major mechanisms used by epidermal cells to participate in immune and inflammatory skin reactions are the production of cytokines and responses to cytokines. Within the epidermis, keratinocytes are the major source of cytokines, along with Langerhans cells and melanocytes (William and Kupper, 1996). Table 1 is a list of known epidermal cell-derived cytokines organized into groups reflecting functions shared by several molecules. Epidermal cells can produce constitutively or following activation, an arsenal of cytokines, strongly supporting the idea that the skin functions as an immune organ and that an important role of the skin is to provide an immune barrier between the external environment and internal tissues. The histopathological pattern of nearly every inflammatory skin disease can be accounted for the appropriate cytokine or combination of cytokines. The pattern of cytokines expressed locally plays a critical role in modulating the nature of tissue inflammation. In this regard, it is important to remember that multiple mechanisms and cell types are involved in the induction of skin toxic responses and determining the source, kinetics of production, and the regulation

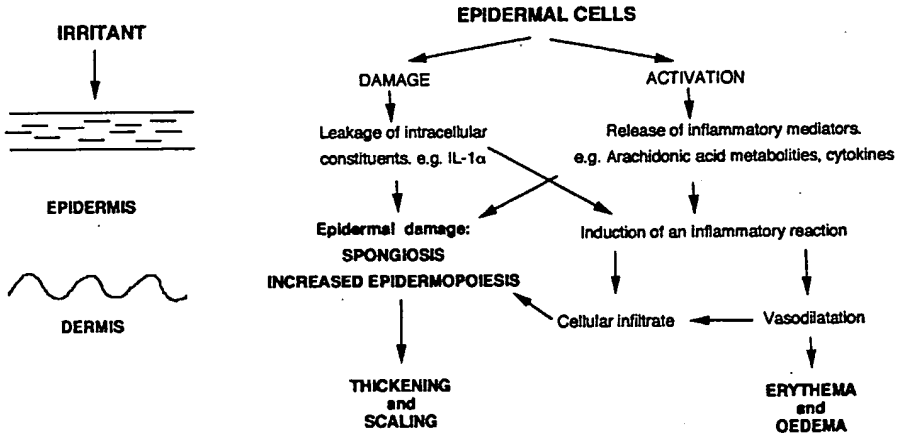

Fig. 1. Sequence of events after application of skin irritant.

Table 1
Epidermal cytokines

Cytokine	Constitutive or inducible expression		
	Keratinocytes	Langerhans cells	Melanocytes
Primary cytokines			
IL-1α	+	+	+
IL-1β	+/−	+	+
TNF-α	+	+/−	+
C-X-C chemokines			
IL-8	+	−	+
Gro-α, Gro-β, Gro-γ	+	+	+
MIP-2 (mouse)	+	+	+
IP-10	+	−	−
C-C chemokines			
MCP-1	+	+	+
MIP-1α	−	+	−
RANTES	+	?	+
Cytokines regulating humoral vs. cellular immunity			
IL-10	+/−	−	+
IL-12	+	+	+
IL-18	+	+	?
Cytokines that promote growth of T cells			
IL-7	+	−	+
IL-15	+	+	?
Cytokine with colony stimulating activity			
IL-6	+	+	+
IL-3 (mice)	+	−	−
G-CSF	+	−	+
M-CSF	+	−	+
GM-CSF	+	+/−	+
Cytokine with growth factor activity for cells other than leukocytes			
TGF-α	+	−	+
EGF	+	?	+
NDF	+	?	+
PDGF	+	?	+
FGF	+	?	+
VEGF	+	?	+
NGF	+	?	+
Immunosuppressive / antagonist cytokines			
IL-1RA	+	?	?
TGF-β	+	+	+
IL-10	+/−	−	+

The informations are taken mainly from reference [3].

of inflammatory mediators in the skin in vivo will be of value in predicting various toxicity arising from exposure to environmental agents.

Keratinocytes represent 95% of epidermal cells, although the primary function of these cells is to provide the structural integrity and barrier function of epidermis, in the last two decades it has become clear that they play an important role in the initiation and perpetuation of skin inflammatory and immunological reactions (McKenzie and Sauder, 1990). While resting keratinocytes produce some cytokines constitutively, a variety of environmental stimuli, such as tumor promoters, ultraviolet light and chemical agents, can induce epidermal keratinocytes to release inflammatory cytokines (IL-1, TNF-α), chemotactic cytokines (IL-8, IP-10), growth promoting cytokines (IL-6, IL-7, IL-15, GM-CSF, TGF-α) and cytokines regulating humoral vs. cellular immunity (IL-10, IL-12, IL-18) (McKenzie and Sauder, 1990).

Of all the cytokines produced by keratinocytes, only IL-1α, IL-1β and TNF-α activate a sufficient number of effector mechanisms to independently trigger cutaneous inflammation (Kupper,

1990). Unstimulated keratinocytes contain large amounts—in biological terms—of preformed and biologically active IL-1α, in addition to inactive pro-IL-1β (Mizutani et al., 1991). Because biologically active IL-1α is produced constitutively by keratinocytes and retained in the cell, the epidermis is a vast reservoir of sequestred IL-1α. Damage to the keratinocyte releases this IL-1α, which essentially is a primary event in skin defense. IL-1α stimulates further release of IL-1α and the production and release of other cytokines such as IL-8, IL-6, GM-CSF, etc. These cytokines are activators for pro-inflammatory cells. In addition to being directly chemotactic for leukocytes, IL-1α induces the expression of intercellular adhesion molecules on the surface of endothelial cells and fibroblasts (Groves et al., 1991). Thus, by cytokine cascades and networks, an inflammatory response can be rapidly generated. Keratinocytes act as pro-inflammatory signal transducers, responding to non-specific external stimuli with the production of inflammatory cytokines, adhesion molecules, and chemotactic factors, preparing the dermal stroma for specific immunological activity. IL-1α stimulates keratinocytes and fibroblast proliferations, thus together with other cytokines, it is also involved in wound healing (reviewed in Kupper and Grove, 1995).

On the other hand, in the skin, TNF-α is stored in dermal mast cells (Gordon and Galli, 1990), but following stimulation it may be produced by keratinocytes (Kock et al., 1990) and Langerhans cells (Larrick et al., 1989). An increasing body of evidence suggests that TNF-α is functionally relevant to a variety of inflammatory skin diseases, both in rodents and humans (Wakefield et al., 1991; Groves et al., 1995). Antibodies against TNF abolishes many inflammatory skin reactions, including allergic and irritant contact dermatitis (Piguet et al., 1991) and graft-vs.-host disease (Piguet et al., 1987). An important mechanism by which TNF influences the development of an inflammatory reaction is induction of the expression of cutaneous and endothelial adhesion molecules (Pober and Contran, 1990; Groves et al., 1995).

Besides 'primary' cytokines, epidermal cells may be induced to produce 'secondary' cytokines, including IL-6, IL-8, and GM-CSF, by 'primary' cytokines or other stimuli, but their secretion, while important in the modulation of skin inflammation, is not sufficient to induce it in the absence of other stimuli (Kupper, 1990).

3. Role of pro-inflammatory cytokines in xenobiotics-induced skin irriation

A wide variety of chemicals can induce skin irritation. Although everybody agrees on the pivotal role of cytokines in skin inflammatory reactions, a limited number of irritants have been tested in vivo for the specific induction of cytokines. Most of these studies have used sodium dodecyl sulfate, benzalkonium chloride, nonanoic acid, phenol, sulfur mustard or croton oil (or its active ingredient tetradecanoylphorbol 13-acetate) as reference irritants (Enk and Katz, 1992; Kondo et al., 1994; Hoefakker et al., 1995; Luster et al., 1995; Grangsjo et al., 1996; Tsuruta et al., 1996; Holliday et al., 1997). As a laboratory, our specific interest focuses on the adverse effects of pesticides. In particular, we have turned our attention to triorganotin compounds. Occupational and accidental exposure to tributyltin (TBT), a biocidal agent used mainly in wood preservation and marine antifouling paints, can result in skin and eye irritation, and severe dermatitis has been reported after direct contact with the skin (WHO, 1990). We have previously demonstrated in the mouse (Corsini et al., 1996a, 1997) the involvement of the pro-inflammatory cytokines IL-1α and TNF-α in TBT-induced skin irritation. Prior treatment with neutralizing antibodies against IL-1α or TNF-α significantly reduced skin irritation. Interestingly, however, as indicated by RT-PCR analysis, TNF-α expression was not affected by prior treatment with IL-1α antibody. Indeed, the kinetics of TNF-α and of IL-1α released from ear slices after treatment with TBT went in parallel, which indicates that TNF-α production induced by TBT is independent of IL-1α release and clearly indicates that IL-1α is only one of the inflammatory mediators involved and not necessarily the only primary event in the skin defence mechanism.

The ability of TBT to induce TNF-α is not limited to this pesticide, but keratinocyte-derived TNF-α mRNA can rapidly be upregulated fol-

lowing treatment with SDS and allergens in mice (Enk and Katz, 1992). Also in our experience sodium dodecyl sulfate and skin allergens, such as dinitrochlorobenzene and oxazolone, all induce TNF-α proteins (Holliday et al., 1997). However, it is not possible to generalize since not all chemicals that have the ability to cause skin irritation will elicit detectable TNF-α responses, as clearly demonstrated by Holliday et al. (1997) with the skin irritant benzalkonium chloride, which failed to induce TNF-α production at concentrations that caused a vigorous inflammatory response in the skin.

It appears therefore that xenobiotics which are known to induce skin irritation differ with respect to their ability to up-regulate pro-inflammatory cytokines and that cutaneous inflammatory reactions are not all dependent on local production of TNF-α. Whether the characteristics of inflammatory response differ from those reactions that are associated with TNF-α remains to be determined.

4. On the mechanisms of xenobiotics-induced IL-1α production in murine keratinocytes

From a toxicological point of view it is very important to understand the mechanism(s) by which chemicals induce the production of cytokines. Many factors can increase in vitro IL-1α production, including LPS, various toxins, cytokines, ultraviolet radiation, and phorbol esters, but the molecular mechanisms responsible for xenobiotics induction of IL-1α production are poorly understood. In this regard we have investigated the mechanisms of TBT-induced IL-1α production. We have focused our attention on IL-1α, since its release form basal keratinocytes is sufficient to both initiate and mediate cutaneous inflammation in vivo (Kupper, 1990). TBT induces a dose and time dependent increase in IL-1α both released and cell-associated (Corsini et al., 1996a). It is well known that triorganotins can disturb mitochondrial activity, binding to a component of the ATP synthase complex, inhibiting mitochondrial ATP synthesis and disturbing the proton gradient (reviewed by Snoeij et al., 1987). This could divert electrons from the respiratory chain into the formation of ROS known to be involved in activation of transcription factors and production of cytokines (Ilnicka et al., 1993; Lee and Ilnicka, 1993; Baeuerle and Henkel, 1994). Inducible expression of IL-1α and TNF-α are controlled by regulation of the activity of transcription factors, mainly NF-κB and AP-1 (Muegge and Durum, 1990; Fenton, 1992). We have previously reported in a murine keratinocyte cell line HEL30 (Corsini et al., 1996b) that TBT promptly activated transcription factor NF-κB prior to IL-1α production and that the depletion of functional mitochondria by long-term treatment with ethidium bromide resulted in a dramatic reduction of TBT-induced NF-κB activation and IL-1α production, which suggests that mitochondria serve as mediators of TBT effects on ROS generation. However, the earliest event triggered by TBT treatment is an increase in intracellular Ca^{2+} during 1–4 min, followed by ROS generation (15 min), mainly at the mitochondrial level, then by NF-κB activation (30 min), and, finally by IL-1α production (4 h). All these events can be partially or totally inhibited by preventing the increase in intracellular Ca^{2+}, whereas scavenging of ROS does not affect the TBT-induced increase in Ca^{2+}. Thus, our results suggest that alterations in Ca^{2+} homeostasis may initiate TBT-induced oxidative stress. High cytoplasmic Ca^{2+} levels can cause an increased mitochondrial Ca^{2+} uptake and disruption of mitochondrial Ca^{2+} homeostasis that results in increased ROS formation (Chacon and Acosta, 1991) due to stimulation of electron flux along the electron transport chain. Beside this primary effect, the oxidative stress can be responsible for cell damage and leakage of cell-associated IL-1α, which can in turn active keratinocytes to produce more IL-1α. Indeed, we could also demonstrate, by using a neutralizing antibody against murine IL-1α, an autocrine effect of IL-1α on its own production in TBT-stimulated cells.

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Rapid communication

In vivo expression of IL-12 and IL-13 in atopic dermatitis

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Previous studies in atopic dermatitis (AD) have shown that acute and chronic skin lesions are associated with a TH_2 -type profile of cytokine expression. IL-12 and IL-13 are recently described cytokines, which possess TH_1 - and TH_2 -like actions, respectively. We have used the technique of in situ hybridization to examine the expression of IL-12 and IL-13 messenger RNA in skin biopsy specimens of acute and chronic skin lesions and uninvolved skin from patients with AD. When compared with normal control skin, the acute and chronic skin lesions and unaffected skin from patients with AD had significantly greater numbers of cells that were positive for IL-13 mRNA ($p < 0.05$). Acute AD skin lesions expressed a higher number of positive cells than those observed in chronic AD skin lesions ($p < 0.05$) or psoriasis skin lesions ($p < 0.05$) There was a significant increase in the numbers of IL-12 mRNA-positive cells in chronic skin lesions compared with acute lesions and uninvolved skin from patients with AD ($p < 0.05$). These data demonstrate that acute AD skin lesions are associated with an increased expression of IL-13 mRNA. In contrast, the relative increase in IL-12 mRNA in chronic AD skin lesions suggests a possible role for IL-12-producing cells in modulating chronic inflammation.

Key words:

Atopic dermatitis

IL-12

IL-13

allergic inflammation

T lymphocytes

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Abbreviations used

AD:
Atopic dermatitis
ANOVA:
Analysis of variance
IFN-gamma:
Interferon-gamma

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Atopic dermatitis (AD) is a chronic inflammatory skin disease of unknown etiology that is associated with elevated serum IgE levels and tissue eosinophilia. [1] Skin biopsy specimens of acute and chronic lesions in AD are characterized by an infiltration with a large number of inflammatory cells, in particular $CD4^+$ T lymphocytes. [2] We and others have shown that there is a high expression of IL-4, IL-5, and IL-10 but not interferon-gamma (IFN-gamma) in both acute and chronic lesions of AD

Abbreviations used
AD: Atopic dermatitis
ANOVA: Analysis of variance
IFN-γ: Interferon-γ

compared with the uninvolved skin of patients with AD or normal control subjects. [3] [4] These observations suggest the expression of predominantly TH_2 -type cytokines in AD, which are critical for IgE synthesis and eosinophil accumulation in skin lesions.

IL-12 and IL-13 are recently described cytokines, which play a critical role in the development of TH_1 - versus TH_2 -like T cells, respectively. Although the primary source of IL-12 is macrophages or monocytes, because of its spectrum of biologic activity in stimulating the induction of TH_1 cells [5] and inhibiting IgE production, [6] IL-12 is considered to be a TH_1 -type cytokine. IL-13 is produced mainly by activated T cells of the TH_2 subset,

which shares a number of biologic activities with IL-4. [7] In this study we have investigated the expression of IL-12 and IL-13 in AD to compare the expression of these cytokines in acute lesions with chronic lesions, uninvolved skin of patients with AD, psoriatic skin lesions, or skin of normal control subjects by using the technique of in situ hybridization.

METHODS

Skin biopsy specimens

Fifteen punch skin biopsy specimens were obtained from five patients who met the diagnostic criteria for AD. [1] [3] Three biopsy specimens were obtained from each patient, one specimen from acute,

erythematous AD lesions of less than 3 days' onset, a second specimen from chronic lichenified AD lesions of greater than 2 weeks' duration, and a third specimen from uninvolved skin. The clinical severity of each skin lesion was graded according to previously described criteria. [3] Control skin biopsy specimens were also obtained from nonatopic normal volunteers ($n = 5$) and affected skin from patients with psoriasis ($n = 4$). Skin biopsy specimens were fixed immediately and prepared for in situ hybridization. [3]

The age of patients with AD ranged from 19 to 49 years. All patients had a history of AD dating to early infancy and had associated respiratory allergy (allergic rhinitis and/or asthma). Serum IgE levels ranged from 269 to 10,700 IU/ml. None of these patients had other skin conditions, and none had previously received oral steroids. Topical steroids were withheld for at least 2 weeks before biopsies were performed.

In situ hybridization

In situ hybridization was performed as previously described. [8] Briefly, riboprobes (both antisense and sense) were prepared from complementary DNA and linearized with appropriate enzymes before transcription. The IL-12 probes used were specific for the p35 or the p40 subunits of the IL-12 gene. Transcription was performed in the presence of sulfur 35-labeled uridine triphosphate and the appropriate T7 or SP6 RNA polymerases. Cryostat sections were permeabilized and subsequently treated to prevent nonspecific binding of the ^{35} S-labeled RNA probes. [3] Prehybridization was carried out with 50% formamide and 2? standard saline citrate for 15 minutes at 40? C. Dithiothreitol was included in the hybridization mixture to ensure blocking of any further nonspecific binding of the ^{35} S-labeled probes. Posthybridization washing was performed in decreasing concentrations of standard saline citrate at 45? C. Unhybridized single-strand RNAs were removed by RNase A. Hybridization signals were visualized by using autoradiography, and the tissue sections were counterstained with hematoxylin.

Quantification

Slides were coded and counted blindly by using 100? magnification with an eyepiece graticule. The results were expressed as the mean number of positive cells per field. The number of fields per section counted was two to six, depending on the size of the biopsy specimen and the pattern of alignment of the grid covering an intact area of epithelial and subepithelial tissue. The intra-observer coefficient of variation for repeated measures was less than 5%. [9]

For the negative controls, skin biopsy specimens were hybridized with sense probes. In addition, sections were treated with RNase A solution before the prehybridization step with antisense probes. Positive cells were only observed when the antisense probes were used; preparations treated with the sense probes or pretreated with RNase were negative with only baseline background signals.

Statistical analysis

Statistical comparison of the results was performed by using an analysis of variance (ANOVA), and subsequent post hoc comparisons were made by using a Tukey HSD multiple comparison test (Systat v 5.1; Systat Inc., Evanston, Ill.). A p value of less than 0.05 was considered statistically significant.

RESULTS

Biopsy specimens from acute AD lesions demonstrated varying degrees of epidermal hyperplasia with focal spongiosis and epidermal inflammatory infiltrate. Perivascular infiltrates were also prominent. In contrast, chronic AD lesions were characterized by acanthosis with minimum epidermal infiltration. All biopsy specimens from acute and chronic AD skin lesions showed positive hybridization signals for IL-13 mRNA (Fig. 1) . Biopsy specimens from uninvolved skin obtained from patients with AD demonstrated a few IL-13 mRNA-positive cells. Only two of five of the normal skin biopsy specimens and two of four of the psoriasis skin biopsy specimens were positive for IL-13 mRNA, but the numbers of positive cells were very few. There was no significant difference in the numbers of IL-13

mRNA-positive cells between patients with psoriasis and normal control subjects ($p > 0.05$). The number of IL-13 mRNA-positive cells was significantly higher in acute lesions compared with chronic lesions ($p < 0.05$), uninvolved skin ($p < 0.001$), normal skin ($p < 0.001$), or skin lesions from patients with psoriasis ($p < 0.05$). However, the number of IL-13 mRNA-positive cells in chronic lesions was still higher than the number of IL-13 mRNA-positive cells in uninvolved skin and normal skin ($p < 0.01$) and in

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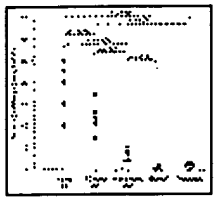

Figure 1. In situ hybridization of AD skin lesions, uninvolved skin, normal skin, and skin biopsy specimens from patients with psoriasis when riboprobes for IL-13 were used. Results are expressed as the number of positive cells per high-power field (0.202 mm^2). Comparisons were made by using a nonparametric ANOVA test. IL-13 mRNA-positive cells were significantly increased in AD skin lesions and uninvolved skin when compared with normal control skin and psoriatic skin lesions ($p < 0.05$).

psoriatic skin lesions ($p < 0.05$). Although there were few IL-13 mRNA-positive cells present in uninvolved skin, the number was significantly higher than that in normal skin ($p < 0.05$). In general, the mRNA-positive cells for IL-13 were located among the inflammatory infiltrates, mainly at the upper part of the dermis.

The expression of IL-12 mRNA was variable in all the groups studied (Figs. 2 and 3) . When both p35 and p40 probes were used, the differences in the number of cells expressing IL-12 mRNA in acute lesions, compared with uninvolved or normal skin, were statistically nonsignificant. However, the number of IL-12 mRNA-positive cells in chronic skin lesions, as detected by the p40 mRNA probe, was significantly higher than the number of positive cells in acute AD lesions. When the p35 IL-12 mRNA probe was used, the difference between the numbers of positive cells in chronic and acute skin lesions just failed to reach statistical significance ($p < 0.08$). When both the p35 and p40 probes were used, the number of IL-12 mRNA-positive cells in chronic AD lesions was significantly higher than that in uninvolved skin ($p < 0.05$). However, only when using the p40 probe was the expression of IL-12 mRNA in chronic skin lesions significantly higher than that in normal skin ($p < 0.05$). In contrast to IL-13, IL-12 mRNA-positive cells were demonstrated in all biopsy specimens from normal control subjects, uninvolved skin of patients with AD, and skin samples from patients with psoriasis. The numbers of IL-12 mRNA-positive cells were significantly elevated in skin biopsy specimens from patients with psoriasis when compared with normal control subjects ($p < 0.05$). Morphologically, the IL-12 mRNA cells were a mixture of large cells that consisted of tissue macrophages and smaller cells. Few IL-12 or IL-13 mRNA-positive cells were demonstrated in the epidermis, but their structure was consistent with infiltrating inflammatory cells rather than keratinocytes. There was no significant hybridization signal in skin sections from the different groups with the use of the radiolabeled sense IL-12 or IL-13 probes or when the preparations were treated with RNase before the application of the antisense

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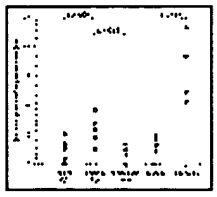

Figure 2. In situ hybridization of AD skin lesions, uninvolved skin, normal skin, and skin biopsy specimens from patients with psoriasis when the p35 riboprobe for IL-12 was used. Results are expressed as the number of positive cells per high-power field (0.202 mm^2). Comparisons were made by using a nonparametric ANOVA test. IL-12 (p35) mRNA-positive cells were significantly increased in chronic AD skin lesions when compared with uninvolved skin ($p < 0.05$) and in biopsy specimens from patients with psoriasis compared with normal skin ($p < 0.05$).

probe, confirming the specificity of the probes and signals.

DISCUSSION

We and others have recently reported that acute AD, in common with other forms of atopic inflammation, is characterized by infiltration of the skin by cells expressing predominantly TH_2 -like cytokines, such as IL-4, IL-5, and IL-10. [3] [10] To extend our investigations into the pathogenesis of AD and assess further differences in cytokine expression between acute and chronic skin lesions, we examined mRNA for IL-12 and IL-13 in skin biopsy specimens from patients with AD and compared them with skin biopsy specimens from patients with psoriasis and nonatopic normal skin.

Our results provide direct evidence for the predominant expression of IL-13 in AD and support the role of TH_2 -type cytokines in the pathogenesis of this disease. This study demonstrates that both acute and chronic AD lesions are associated with an increased expression of IL-13 mRNA when compared with skin biopsy specimens from normal control subjects. These results cannot be attributed to a nonspecific accumulation of lymphocytes within the skin because biopsy specimens from patients with psoriasis did not show enhanced numbers of IL-13 mRNA-positive cells.

Similar elevated levels of IL-13 mRNA expression have recently been reported by our laboratory in bronchial biopsy specimens from patients with allergic asthma. [11] These observations support an active role for IL-13 in the pathogenesis of allergic disorders. Interestingly, in patients with allergic asthma, levels of mRNA for IL-13 are subsequently reduced after steroid therapy in patients who showed a clinical improvement in response to steroids. [11] This further strengthens the association between the development of atopic diseases and the synthesis of IL-13.

In this study we did not attempt to identify the phenotype of cells responsible for the synthesis of mRNA for IL-13. Both $CD4^+$ and $CD8^+$ T cells have been reported as having the capacity to produce IL-13. [7] However, it has recently been demonstrated that activated mast cells, and possibly basophils, also possess the capacity to secrete IL-13. [12] Although we did not attempt to quantify

Figure 3. In situ hybridization of AD skin lesions, uninvolved skin, normal skin, and skin biopsy specimens from patients with psoriasis when the p40 riboprobe for IL-12 was used. Results are expressed as the number of positive cells per high-power field (0.202 mm^2). Comparisons were made by using a nonparametric ANOVA test. IL-12 (p40) mRNA-positive cells were significantly increased in chronic AD skin lesions when compared with acute skin lesions ($p < 0.05$), uninvolved skin ($p < 0.05$) and in skin biopsy specimens taken from normal individuals ($p < 0.05$). IL-12 mRNA expression was also increased in biopsy specimens from patients with psoriasis when compared with normal skin ($p < 0.05$).

IL-4 mRNA-positive cells in these biopsy samples, we have previously reported the numbers of cells positive for IL-4 mRNA in acute and chronic AD. [3] The results presented in this article suggest that a similar number of cells positive for IL-4 and IL-13 mRNA is observed in both groups. Furthermore, in a recent study on bronchial biopsy specimens from patients with allergic asthma, we have shown that IL-13 is colocalized with IL-4, and both are expressed in $CD3^+$ cells. [13] We therefore believe that most of the IL-13 mRNA-positive cells in AD are resident or infiltrating T cells.

The highest number of IL-13 mRNA-positive cells was observed in the acute skin lesions, and this number was significantly greater than that seen in chronic skin lesions. A differing profile of cytokine gene expression has previously been documented in acute and chronic AD lesions, [3] and these observations may be related to the differences in histopathology noted in this study and reported previously. [14] IL-13 shares many biologic properties with IL-4, including the ability to act as a switch factor inducing the production of IgE via a common signalling pathway. [15] [16] IL-13 has also been shown to downregulate the production of IL-12 and IFN-gamma, [7] and the presence of this cytokine may be one explanation for the relative paucity of IL-12 mRNA-positive cells in acute AD. In contrast, chronic lesions were found to be associated with relatively few cells expressing mRNA for IL-4 and significantly greater numbers of IL-5 mRNA-positive cells and activated eosinophils. Because IL-13 has recently been shown to selectively induce vascular cell adhesion molecule-1 (an adhesion molecule associated with eosinophil accumulation) expression on human endothelial cells, [17] it is likely that this

cytokine plays a role in the recruitment of these cells to the sites of AD skin lesions. Whether acute and chronic lesions represent different stages of an ongoing allergic inflammatory response with a common pathogenesis or whether they each have a distinct immunologic basis remains to be determined.

A significantly elevated number of cells expressing

mRNA for IL-13 was also observed in uninvolved skin from patients with AD compared with normal control subjects. Thus enhanced synthesis of IL-13 may reflect an atopic disposition of the skin, possibly being primed for the production of IgE antibodies. Whether this observation is restricted to patients with allergic cutaneous responses or is evident in atopic subjects in general is presently unknown.

There was considerable variability in the numbers of IL-12 mRNA-positive cells in all skin biopsy specimens studied, although there was a similar pattern of mRNA expression when both p35 and p40 subunits of the IL-12 molecule were examined. It has been previously shown that the p35 subunit of IL-12 is expressed constitutively by cells, whereas the p40 subunit is inducible. [18] The expression of both of these subunits is required for biologic activity. Because the expression of both subunits was elevated in chronic AD skin lesions, our data suggest that the biologically active heterodimer form of IL-12 was being produced. There was no significant difference in the number of IL-12 mRNA-positive cells in biopsy specimens from patients with acute AD compared with specimens from normal control subjects or uninvolved skin. The number of IL-12 mRNA-positive cells was significantly higher in chronic AD lesions, when compared with acute AD lesions, uninvolved skin, and biopsy specimens from normal subjects. Biopsy specimens from patients with psoriasis demonstrated large numbers of IL-12 mRNA-positive cells. We believe that most of the IL-12 mRNA-positive cells are macrophages, because the positive cells were relatively large. Moreover, we have recently reported the phenotype of IL-12 mRNA-positive cells in cutaneous late-phase reactions after immunotherapy, where we demonstrated that 80% of the cells positive for IL-12 mRNA were macrophages. [19] The relative increase in the IL-12 mRNA-positive cells in chronic lesions might reflect the ability of macrophages to regulate and control the inflammatory response in AD. IL-12 has the ability to suppress IgE production in vitro by both IFN-gamma-dependent and IFN-gamma-independent mechanisms [6] and can switch TH_0 cells to a TH_1 -like phenotype. [5] These data are consistent with our finding of IL-12 expression after successful treatment with steroids [11] or after immunotherapy. [19] IL-12 is also a potent inducer of IFN-gamma production, [20] although in chronic AD lesions no significant increase in the production of IFN-gamma has been observed. [3] This lack of IFN-gamma production may be explained by the increased expression of IL-4, IL-10, and prostaglandin E_2 observed in AD, [3] [21] [22] all of which serve to limit the synthesis of IFN-gamma.

In summary, we have demonstrated enhanced expression of mRNA for IL-13 in acute and chronic skin lesions in patients with AD and, to a lesser extent, in the uninvolved skin from these individuals. The numbers of IL-12 mRNA-positive cells were primarily elevated in chronic skin lesions. These data suggest that IL-13 might play a role in the pathogenesis of AD and support the hypothesis that inflammation in acute AD is mediated by TH_2 -type cytokines. It also opens a new avenue for the design of new therapeutic approaches in this common skin disease.

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Overexpression of IL-10 in Atopic Dermatitis

Contrasting Cytokine Patterns with Delayed-Type Hypersensitivity Reactions[1]

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The skin lesions of patients with atopic dermatitis provide a model to study immunoregulation in human allergy. To determine the local cytokine pattern of cells present (both endogenous and recruited) at the site of disease, we extracted RNA from skin biopsy specimens from patients with atopic dermatitis, allergic contact dermatitis, and positive tuberculin reactions and used PCR to assay for cytokine mRNA. cDNAs were normalized to the intensity of the CD3δ PCR product as a marker of T cell mRNA. We found overexpression of IL-10 mRNA in atopic dermatitis lesions, in comparison with allergic contact dermatitis lesions and tuberculin reactions. In contrast, IL-4 mRNA was most strongly expressed in allergic contact dermatitis lesions and IFN-γ mRNA was the predominant cytokine in tuberculin reactions. Using an anti-IL-10 mAb with immunoperoxidase, we localized IL-10 protein to large mononuclear cells in the dermal infiltrate of atopic lesions. After immunomagnetic sorting of mononuclear cell populations from PBMC of atopic dermatitis subjects, IL-10 mRNA as measured by PCR was found to be strongly expressed in $CD14^+$ cells. Spontaneous release of IL-10 from PBMC-derived adherent cells was greater in atopic dermatitis donors than normal controls. We therefore renormalized skin biopsy cDNA according to the level of β-actin PCR product, as a marker of total cellular mRNA, and found by PCR that IL-10 was nevertheless greatest in atopic dermatitis subjects. We conclude that the relative overexpression of IL-10 in atopic dermatitis may contribute to the up-regulation of humoral responses and the down-regulation of Th1 responses. *The Journal of Immunology*, 1995, 154: 1956–1963.

Atopic dermatitis is a chronic inflammatory disease that manifests as eczematous skin lesions. The association of atopic dermatitis with allergic respiratory diseases and the presence of elevated serum IgE levels has suggested an allergic etiology. Yet the inflammatory infiltrate within atopic dermatitis skin lesions is characterized by a predominance of $CD4^+$ T cells (1). This apparent paradox, $CD4^+$ T cells predominating at the site of disease with B cell activation as a critical component, has been reconciled with the introduction of the Th1-Th2 paradigm of immune regulation.

At least two subpopulations of T cells, differentiated on the basis of the pattern of the cytokines they produce, regulate cell-mediated and humoral responses (2). T cells producing the Th1 profile of cytokines, typified by IFN-γ, are critical to cell-mediated immunity required to eliminate some bacterial pathogens and protozoan parasites (3–8). In contrast, T cells producing the Th2 profile of cytokines, typified by IL-4, are involved in humoral immunity that may be critical for regulating responses to helminth parasites (9, 10). Recent investigations have indicated that Th2 responses participate in allergic disease, with IL-4 detected in T cells from the bronchi of patients with asthma (11) and T cells from the blood and lesions of patients with

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0022-1767/95/$02.00

Table I. *Source of skin biopsy specimens*

Sample	Patient	Diagnosis	Age	Sex	Duration	Location
AD1	A	Atopic dermatitis	57	M	53 yrs	Right thigh
AD2	B	Atopic dermatitis	41	M	41 yrs	Nuchal
AD3	C	Atopic dermatitis	28	M	28 yrs	Back
AD4	C	Atopic dermatitis	28	M	28 yrs	Left leg
AD5	D	Atopic dermatitis	53	F	53 yrs	Antecubital
AD6	E	Atopic dermatitis	56	M	56 yrs	Left anterior chest
CD1	F	Patch test	48	M	48 h	Volar forearm
CD2	F	Patch test	48	M	48 h	Volar forearm
CD3	G	Patch test	24	M	48 h	Back
CD4	H	Poison ivy	24	M	24 h	Lower back
CD5	I	Poison ivy	28	M	24 h	Lower back
CD6	J	DNCB[a]	34	F	48 h	Volar forearm
CD7	K	DNCB	52	M	48 h	Volar forearm
CD8	J	DNCB	34	F	96 h	Volar forearm
CD9	K	DNCB	52	M	96 h	Volar forearm
PPD1	L	PPD with pulmonary TB[b]	70	M	48 h	Left arm
PPD2	L	PPD with pulmonary TB	70	M	96 h	Left arm
PPD3	M	PPD with pulmonary TB	53	M	48 h	Left arm
PPD4	M	PPD with pulmonary TB	53	M	96 h	Left arm

[a] DNCB, dinitrochlorobenzene.
[b] TB, tuberculosis.

atopic dermatitis (12–17). In allergic disease, IL-4 may up-regulate IgE production against exogenous stimuli.

The objective of the present study was to more fully characterize the Th1-Th2 cytokine pattern in human allergic conditions. We measured several cytokine mRNA in tissue lesions of patients with atopic dermatitis to provide insight as to the nature of immune response at the local site of disease activity. For comparison, we simultaneously measured cytokine mRNA in tuberculin reactions, a standard DTH[3] response, and in allergic contact dermatitis sites, also thought to represent DTH.

Materials and Methods

Subjects

Skin biopsy specimens were obtained after informed consent from five patients with atopic dermatitis, six patients with contact dermatitis and two patients with pulmonary tuberculosis as outlined in Table I. Patients with atopic dermatitis had chronic moderate to severe disease with typical features. Peripheral blood was obtained from patients with atopic dermatitis and normal controls that had no personal history of atopic disease.

PBMC isolation

PBMC were isolated on Ficoll-Paque (Pharmacia Biotech Inc., Piscataway, NJ) gradients. Cells were immunomagnetically separated by using Dynabeads (Dynal International, Skoyen, Norway). Cells were incubated (30 min at 4°C) with beads directly coated with specific lymphocyte mAbs (CD4, CD8, or CD19) and positive cells were recovered magnetically. Monocytes were isolated similarly by a two-step method using anti-CD14 (anti-Leu M3, Becton Dickinson, San Jose, CA) followed by sheep anti-mouse Ig-coated beads. Flow cytometry demonstrated enrichment for the selected cell populations (89% CD4, 98% CD8, 97% CD19, and 82% CD14). Purity of cell populations was also demonstrated by PCR detection of CD3δ mRNA as described (3, 18). CD3δ was detected in the $CD4^+$ and $CD8^+$ T cell populations only.

[3] Abbreviation used in this paper: DTH, delayed-type hypersensitivity; PPD, purified protein derivative.

RNA isolation and cDNA synthesis

Total RNA was isolated from biopsy specimens or mononuclear cells by the method of Chomczynski and Sacchi (19). To facilitate the rapid lysis of the cells isolated from tissue, forty consecutive 5-μm-thick cryostat sections were added to 4 M guanidinium isothiocyanate buffer. After RNA extraction, the samples were treated with DNase 1 (Promega Corp., Madison, WI) for 30 min at 37°C. RNase inhibitor (Boehringer Mannheim Corp., Indianapolis, IN) was present during all enzymatic manipulations of RNA. cDNA was synthesized from oligo-dT-primed RNA by incubation at 42°C with Maloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). For comparison of cytokine mRNA, cDNA concentrations were normalized to yield equivalent CD3δ or β-actin PCR products as described (3).

PCR

PCR was performed as previously described (3, 18). The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01%(w/v) gelatin, 0.2 mM dNTP, 25 pM 5′ and 3′ oligonucleotide primers, and 2.5 U of Taq polymerase (Promega). Aliquots were then amplified by 35 cycles in a GeneAmp 9600 DNA thermocycler (Perkin-Elmer Corp., Irvine, CA). Each cycle consisted of denaturation at 94°C for 40 s and annealing/extension at 65°C for 1 min. An aliquot of PCR product was then electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. The sequences of cytokine-specific primer pairs, 5′ and 3′, have been previously reported (18). The sequences of the IL-10 primers are specific for human IL-10 and are not homologous with viral IL-10 sequences.

Radioactive hybridization of PCR product

To verify PCR results, PCR products were electrophoresed and transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL). An oligonucleotide complementary to sequences internal to the sequences recognized by the PCR amplification primers was labeled at the 5′ end by T4 polynucleotide kinase (Boehringer Mannheim) and [^{32}P]γATP (7000Ci/mM; ICN, Costa Mesa, CA) for use as a radioactive probe. Blots were hybridized with probe for 4 h, washed for 5 min with 2X SSC and 0.1% SDS, followed by 0.2X SSC and 0.5% SDS at ambient temperature, and then exposed to x-ray film. Sequences of the oligonucleotide probes have been previously reported (18).

PCR products were quantified by using an AMBIS radioanalytic imaging system (Automated Microbiology Systems, San Diego, CA). Gels were scanned and the amount of radioactivity bound to PCR products

present in any one lane was determined. The relative intensity of individual bands on a given gel was expressed as a percentage of that band with the maximal cpm in each experiment for that particular cytokine.

Validity of PCR

PCR analysis of 10-fold serially diluted plasmids containing cytokine cDNA with visualization by ethidium bromide staining indicated that our PCR procedure was sensitive to the order of 10^2 to 10^3 copies for each cytokine (18). Furthermore, the intensity of the PCR product increased according to the number of copies of starting plasmid to at least 10^6 copies. There was a log-linear correlation between the number of starting copies and the quantity of PCR products throughout the range investigated. These results indicate that our PCR conditions provide meaningful comparison of the small amounts of cytokine mRNA present in lesions. A number of controls were employed to ensure accurate comparisons of the different samples studied as previously outlined (18). Cytokine levels were compared with that of normal skin, in which mRNA could not be detected with the number of cycles used (data not shown).

IL-10 protein detection by immunohistochemistry

IL-10 expression in biopsy specimens was determined by immunoperoxidase labeling of cryostat sections with rat anti-IL-10 (clone JES3–9D7, 10 μg/ml; PharMingen, San Diego, CA) mAb or an isotype-matched control mAb (20). Skin biopsy specimens were embedded in OCT medium (Ames Co., Elkhart, IN) and snap frozen in liquid nitrogen. The tissues were stored at −70°C until sectioning. Sections (3 to 5 μm) were acetone fixed and blocked with normal goat serum before undergoing incubations with the mAbs (120 μg/ml) for 60 min followed by biotinylated goat anti-rat IgG for 30 min. Slides were washed with phosphate buffer between incubations. Primary Ab was visualized with the ABC Elite system (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin and mounted in aqueous dry mounting medium (Crystal Mount, Biomeda Corp., Foster City, CA).

Monocyte isolation

Adherent cells were obtained by allowing PBMC (4×10^6 cells/ml in RPMI with 10% heat-inactivated FCS) to adhere in a 16- × 100-mm petri dish for 2 h at 37°C. The nonadherent cells were washed away three times with warm RPMI and the adherent monocytes were cultured in RPMI with 10% FCS. After 24 h, supernatants were removed and assayed for IL-10 by ELISA. Cell viability, monitored by trypan blue exclusion was always ≥ 98%. Monocyte purity in the adherent cell preparations, confirmed by α-naphthylacetate esterase and factor XIII immunoperoxidase staining was ≥ 95%.

Detection of IL-10 by ELISA

Detection of human IL-10 protein by sandwich ELISA was performed with rat mAb specific for human IL-10 (PharMingen) according to the manufacturer's protocol. Briefly, 96-well ELISA plates (Corning, Corning, NY) were coated overnight at 4°C with 50 μl per well of the rat anti-human IL-10 mAb at a final concentration of 2 μg/ml. Plates were blocked with 100 μl of 10% FCS in PBS and a 50-μl aliquot of each sample was added to each well. Samples were incubated at room temperature for 1 h, and standard dilutions for rIL-10 (R&D Systems, Minneapolis, MN) were also evaluated. Biotinylated rat anti-human IL-10 mAb (1 μg/ml) was added to each well and incubated for 30 min at room temperature. Avidin-peroxidase (2 mg/ml; Sigma Chemical Co., St. Louis, MO) was added to each well and incubated for 30 min. Substrate solution containing 3-ethylbenzthiazoline-6-sulfonic acid (Sigma Chemical Co.) was added to wells and allowed to undergo color reaction. Plates were read in an ELISA reader (Biotech Instruments, Luton, UK) at a wavelength of 490 Å. Values for IL-10 were calculated from a standard curve of recombinant human IL-10. Statistical analysis was performed by Student's *t*-test.

Results

IFN-γ mRNA in lesions

To evaluate the cytokine pattern in atopic dermatitis lesions, we compared mRNA levels for various cytokines to a standard DTH response, to the tuberculin reaction, and to allergic contact dermatitis. For comparison, cDNA concentrations were normalized to yield equivalent CD3δ PCR products as a marker of T cell mRNA (data not shown). Initially, we examined the level of IFN-γ expression, as this cytokine is a marker of the type 1 pattern associated with DTH.

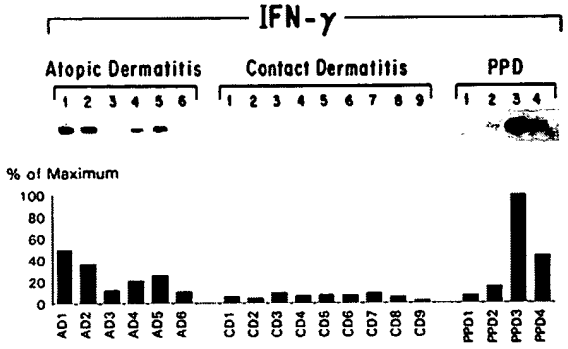

FIGURE 1. IFN-γ mRNA in atopic dermatitis, allergic contact dermatitis, and tuberculin reactions. The hybridized PCR products are shown alongside the PCR intensity expressed as a percent of the maximum cpm.

As expected, we were able to detect IFN-γ mRNA expression in all four of the tuberculin reactions studied, with the levels of IFN-γ mRNA greater than 10% of maximum in three of four specimens (Fig. 1). IFN-γ was weakly expressed in the contact dermatitis lesions, with expression less than 10% of maximum in seven of nine specimens. Surprisingly, we detected IFN-γ mRNA in the atopic dermatitis specimens, with expression greater than 10% of maximum in all six specimens, although the radioactivity detected for subject AD3 may be due to emission from the adjacent lanes.

IL-4 mRNA in lesions

As IL-4 has been reported to be involved in the pathogenesis of atopic dermatitis, we next examined its local production in lesions (Fig. 2). IL-4 was not detected in any of the PPD reactions and was only weakly detectable in the six atopic dermatitis lesions. Strikingly, IL-4 mRNA could be detected visually in seven of nine contact dermatitis specimens, with strong expression, greater than 35% of maximum, in six of nine biopsies. In some instances, a small level of radioactivity was detected in lanes immediately adjacent to strong emitters.

IL-10 mRNA in lesions

Given that the pathogenesis of atopic dermatitis is considered to involve the production of Th2 or type 2 cytokines, we were surprised with the relatively low levels of IL-4 mRNA expression. We therefore decided to examine the local expression of IL-10, another member of the type 2 cytokine pattern (Fig. 3). As anticipated, there was weak

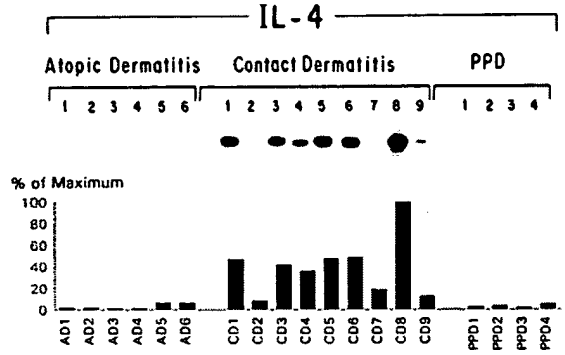

FIGURE 2. IL-4 mRNA in lesions. The IL-4 PCR product and quantified PCR intensity are shown for the atopic dermatitis, allergic contact dermatitis, and tuberculin reactions studied.

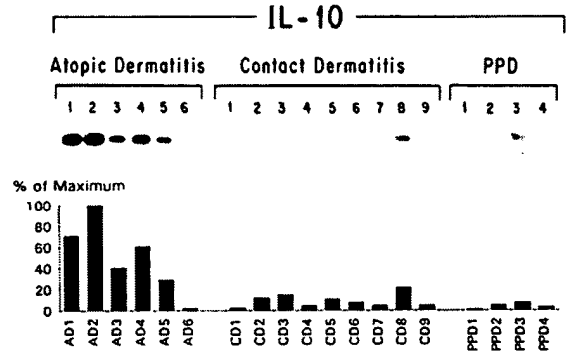

FIGURE 3. IL-10 mRNA in lesions. The IL-10 PCR product and quantified PCR intensity are shown for the atopic dermatitis, allergic contact dermatitis, and tuberculin reactions studied.

expression of IL-10 mRNA in the PPD reactions and contact dermatitis reactions and slightly greater than 10% of maximum in two of four tuberculin reactions and in four of nine contact dermatitis lesions. However, IL-10 mRNA was expressed in five of six atopic dermatitis specimens (greater than 29% of the maximum).

IL-2 and IL-5 mRNA in lesions

To complete the T cell cytokine profiles in the various lesions, we also measured the levels of IL-2 and IL-5 mRNA (Fig. 4). IL-2 was detected in all of the groups studied, expressed at levels greater than 10% of maximum in six of six atopic dermatitis lesions, eight of nine contact dermatitis lesions, and three of four tuberculin reactions. IL-5 was expressed more variably, present at levels greater than 10% of maximum in two of six atopic dermatitis lesions, five of nine contact dermatitis lesions, and none of the tuberculin reactions.

Cellular source of IL-10

To identify the cellular source of IL-10 in subjects with atopic dermatitis, cryostat sections of three biopsy speci-

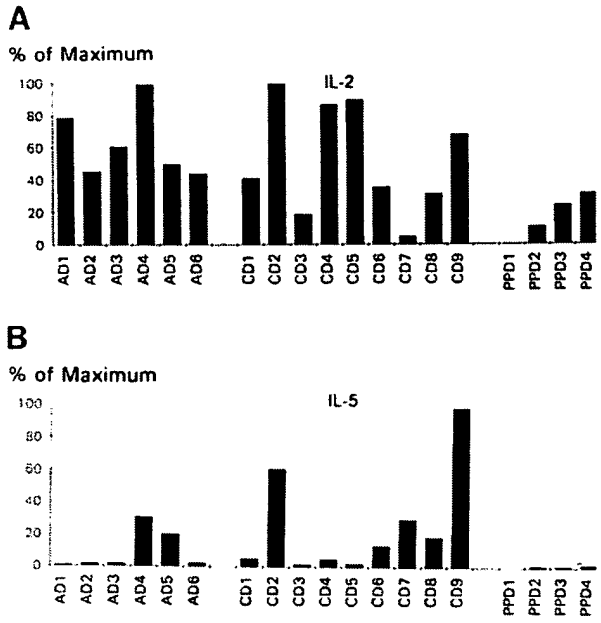

FIGURE 4. IL-2 and IL-5 mRNA in lesions. The PCR intensity, expressed as the percentage of maximal cpm, is shown for the samples studied.

mens of atopic dermatitis were studied by using anti-IL-10 mAbs in conjunction with immunoperoxidase. We found that the positive cells exhibited cytoplasmic staining, were large and oval, and were localized to the dermal perivasuclar infiltrate, suggesting they are monocytes (Fig. 5). These positive cells accounted for approximately 5% of the infiltrating cells. In contrast, no staining was observed in the overlying epidermis.

To further identify the cellular source of IL-10, PBMC from two donors were immunomagnetically sorted into T cells, B cells, and monocyte subsets. After separation, RNA isolation and cDNA synthesis, samples were normalized according to the level of the β-actin mRNA. IL-10 mRNA was detectable in the $CD4^+$, $CD8^+$, and $CD19^+$ populations, but the level of IL-10 was equal to or greater than in the $CD14^+$ monocyte subset (Fig. 6). Measurement of the β-emission from these blots indicated that the $CD14^+$ population contained the highest level of IL-10 mRNA in both subjects (data not shown). These data suggest that monocytes are an important source of IL-10 in atopic dermatitis subjects.

Production of IL-10 in atopic dermatitis subjects

Having demonstrated overexpression of IL-10 mRNA in atopic subjects, we next wanted to determine whether IL-10 protein is also overexpressed. We therefore assayed for spontaneous IL-10 production in 24-h monocyte cultures from 17 patients with atopic dermatitis, compared with 9 normal subjects. Mean levels were significantly higher in the atopic dermatitis (1062 $\pm$ 253 pg/ml) than in

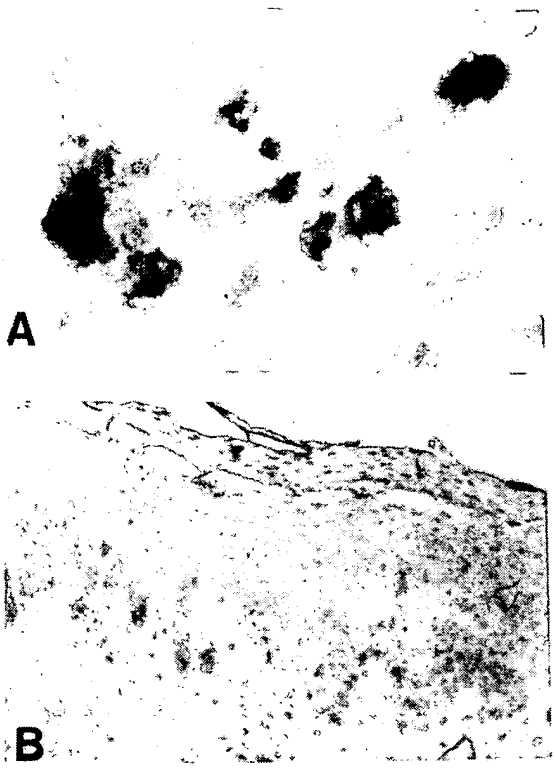

FIGURE 5. Expression of IL-10 in atopic dermatitis lesions. The positive cells are stained black. (*A*) Perivascular infiltrate in the dermis of an atopic dermatitis lesion (magnification ×200). (*B*) Epidermis of the same lesion (magnification ×100). Immunoperoxidase counterstained with hematoxylin.

the normal (563 ± 168, $p = 0.007$) population (Fig. 7). We also measured IL-10 release from monocytes from patients with other dermatoses and found elevated release in 4 of 6 psoriatic and 2 of 4 allergic contact dermatitis patients as compared with normal individuals (data not shown).

Cytokines in atopic dermatitis lesions after normalization to the level of β-actin mRNA

Because our data indicated that IL-10 in atopic dermatitis may have multiple cellular sources, we recompared the levels of IL-10 mRNA within lesions after normalization to β-actin, as a marker of total cellular mRNA. We reanalyzed three samples from the contact dermatitis group, three from the atopic group, and two of the PPD samples for IL-10 mRNA. It was apparent that the greatest levels of IL-10 were present in the atopic subjects (Fig. 8). In contrast, the levels of IL-1β were similar in the atopic dermatitis and contact dermatitis lesions. These data suggest that the overproduction of IL-10 in atopic dermatitis lesions does not simply represent an increased activation of monocytes.

FIGURE 6. IL-10 mRNA expression in cellular subsets of PBMC from atopic dermatitis subjects.

Discussion

Pathogenic concepts of atopic dermatitis include a central role for allergen-specific T cells that produce Th2 or type 2 cytokines, including IL-4 and IL-5. Allergen-specific T cells that produce this cytokine profile have been demonstrated in the peripheral blood and skin lesions of a small number of subjects with active disease (12, 14–17, 21). To further delineate the cytokine profile at the site of disease, we measured cytokine mRNA in skin biopsy specimens from subjects with atopic dermatitis and compared them to tuberculin reactions, a standard DTH response, and biopsies from subjects with allergic contact dermatitis. The striking finding was that each reaction could be differentiated on the basis of the mRNA cytokine pattern: tuberculin reactions characterized by expression of IFN-γ, contact dermatitis by expression of IL-4, and atopic dermatitis by expression of IL-10. We believe that the association of IL-10 with atopic disease is of interest, particularly with regard to monocyte modulation of T helper type 1 T cell function.

We initially assumed that the increased IL-10 expression in atopic dermatitis lesions reflected prominence of T cells with a type 2 or Th0-like cytokine pattern in this disease. However, PCR does not localize the cellular source of mRNA and the level of mRNA expression may not correlate with protein expression. In addition, IL-10 has now been shown to be produced by a variety of cell types apart from T cells, most notably macrophages and keratinocytes (22, 23). Therefore, several cell types may be responsible for IL-10 production in atopic dermatitis. However, we found by immunohistochemical analysis that IL-10 protein was localized to large oval cells resembling monocytes in the dermal perivascular infiltrate of atopic lesions and was not expressed by keratinocytes. IL-10 mRNA was strongly expressed in immunomagnetically sorted $CD14^+$ monocytes from PBMC of atopic subjects. Furthermore, we demonstrated increased spontaneous IL-10 production by atopic monocytes in culture, relative to normal individuals without history of atopic disease. These data indicate that monocytes rather than T cells are the major source of IL-10 in atopic dermatitis and may be important constituents of the immunopathogenesis of this disease. As IL-10 release was also prominent in monocytes from individuals with other dermatoses, the local production of IL-10 in atopic dermatitis likely results from

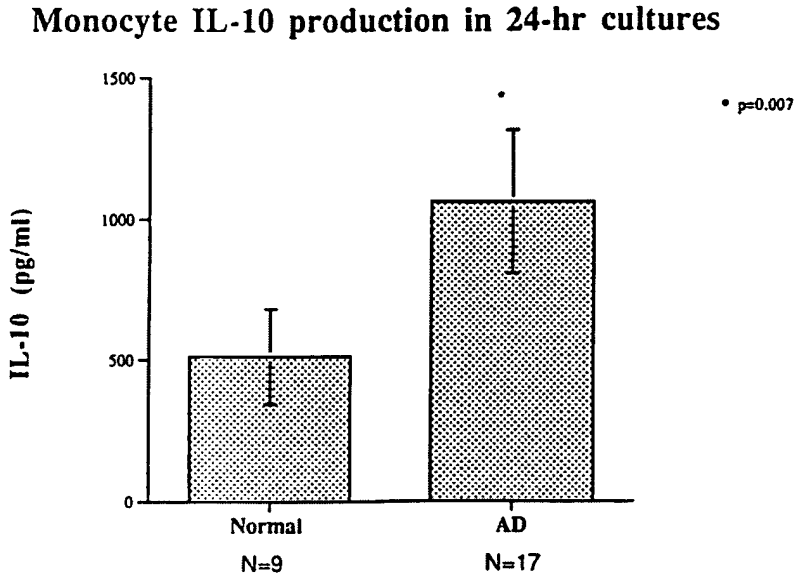

FIGURE 7. Spontaneous release of IL-10 from peripheral blood monocytes in atopic dermatitis subjects and normal controls. Production of IL-10 (pg/ml) by normal and atopic dermatitis (AD) adherent monocytes cultured for 20 h.

a combination of factors including spontaneous release, Ag activation, and the local cytokine milieu. We conclude that additional investigation of the mechanism(s) responsible for IL-10 production in atopic dermatitis should provide insight into the disease pathogenesis.

These results indicating dysregulation of monocyte function in atopic dermatitis are consonant with the recent demonstration that monocytes from subjects with atopic dermatitis also produce increased amounts of prostaglandin E_2 (24). Both IL-10 and PGE_2 inhibit type 1 cytokine production and may account for the decreased IFN-γ production by cultured atopic PBMC (24, 25). Increased IL-10 and prostaglandin E_2 may relate to cAMP-phosphodiesterase activity in atopic monocytes (26). These multiple abnormalities associated with monocytes suggest that this cell may have an important pathogenetic role in immune and inflammatory dysfunction that characterize atopic disease.

It is intriguing to speculate about the role of IL-10 in the pathogenesis of atopic dermatitis. IL-10 induces B cell proliferation and synthesis of IgM, IgG, and IgA with no direct effect on IgE production (27). IL-10 also induces mast cell growth (28) that may be important in the allergic response (29). Furthermore, IL-10 is known to inhibit T cell-mediated reactions, which may account for the reduced allergic contact reactivity in subjects with atopic dermatitis (30). IL-10 is known to inhibit cell-mediated immunity to bacterial pathogens (31) and its production in lesions may contribute to frequent bacterial superinfections such as impetigo. IL-10 may also be responsible for the decreased IFN-γ production in vitro in subjects with atopic dermatitis (24), although IFN-γ production in lesions may be greater than in normal skin (32). In summary, IL-10 may participate in the immune dysregulation that is characteristic of human allergic disease. The data do

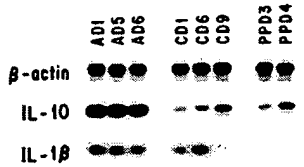

FIGURE 8. Cytokine mRNAs in lesions after normalization to β-actin mRNA.

not exclude a role for IL-4 in atopic dermatitis, which may in the present study appear deceptively low in comparison with allergic contact dermatitis lesions. Additionally, IL-4 may be produced at other sites including lymph nodes and peripheral blood, but the pathogenesis of the skin lesions may be more closely related to the local production of other cytokines such as IL-10.

For a long time now, allergic contact dermatitis has been regarded as a DTH response (type IV immune reaction) and has been used as a comparison for immunologic studies of atopic dermatitis. Yet in 1938, Sulzberger and Baer suggested that "the contact-type of reaction of the human skin is a form of allergy with special and peculiar characteristics which are not necessarily identical with the cutaneous hypersensitivity . . ." (33). They argued that, as opposed to allergic contact dermatitis which involves exposure of the epidermis to allergens, tuberculin hypersensitivity involves the intradermal challenge with foreign protein (34). Furthermore, the two reactions can be differentiated histologically, with a greater degree of epidermal involvement present in contact dermatitis. The present data indicate that the two basic immunologic reactions can also be differentiated on the basis of their local cytokine pattern, with IFN-γ the predominant cytokine in tuberculin hypersensitivity and IL-4 the predominant cytokine in allergic contact dermatitis.

Hauser (35) has reported that Th1 cytokines predominate in murine T cells sensitized in vitro to haptenated APC. In the present study, however, we are examining the effector rather than the induction phase of contact dermatitis. In humans, nickel-specific T cells produce a Th1 pattern (36); however, a urushiol-specific T cell clone produced low levels of Th1 cytokines and behaved as a T suppressor cell in vitro (37), reminiscent of human $CD8^+$ T cells that secrete a Th2-like pattern (4). Additional studies should be undertaken to identify the cellular source of IL-4 in contact dermatitis and whether it is of T cell, B cell, or mast cell origin (38).

The present investigation of the cytokine response in atopic dermatitis provides new data concerning the cytokine profile at the site of disease and indicates a distinct pattern in comparison with classical DTH and allergic reactions. The striking finding was the overexpression of IL-10 in lesions of atopic dermatitis, thus pointing the way for further functional analysis of the role of IL-10 in human allergic disease. These data also suggest that regulation of IL-10 production may be a potential target for immunotherapeutic intervention in atopic dermatitis.

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Epidermal Cytokines IL-1β, TNF-α, and IL-12 in Patients with Atopic Dermatitis: Response to Application of House Dust Mite Antigens[1]

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Epidermal cytokines such as interleukin (IL)-1β, tumor necrosis factor-α, and IL-12 have been described to play a crucial role in the induction and elicitation phase of allergic contact dermatitis upon exposure to haptens. In this study we asked whether these cytokines may also play a role in the epidermis of patients with atopic dermatitis after the application of house dust mite antigens (HDM) to their skin. Epidermal samples were collected by scraping healthy appearing skin of atopic patients and healthy individuals 8 h after the application of an extract of HDM. Sodium lauryl sulfate and saline served as controls. Reverse transcriptase-polymerase chain reaction was performed for IL-1β, tumor necrosis factor-α, IL-12 p35, and IL-12 p40. Exposure to HDM led to a significant upregulation of mRNA of these cytokines in atopic patients only. Whereas IL-1β and tumor necrosis factor-α also showed an upregulation in part of these patients after exposure to the irritant sodium lauryl sulfate, IL-12 p40 mRNA was exclusively enhanced by the application of the allergen. In contrast to IL-12 p40, IL-12 p35 mRNA was not detectable in significant amounts. Interestingly, also in untreated, normal appearing skin of atopic individuals (n = 16), the levels of these cytokines were higher than in normal individuals (n = 8), possibly explaining the increased skin irritability of atopic individuals. Finally, comparing epidermal cytokines in the skin of patients who developed a positive allergen patch test to those who stayed negative, suggests that only expression of IL-1β mRNA may be a predictive marker for the development of a positive patch test reaction to HDM. *Key words: allergen patch test/sodium lauryl sulfate/irritant dermatitis/RT-PCR. J Invest Dermatol 111:1184–1188, 1998*

The allergen patch test with protein antigens such as house dust mite antigen (HDM) and pollen antigens is thought to represent a late type immune reaction of the skin, characteristic for patients with atopic dermatitis (AD) (Clark and Adinoff, 1989). It has been widely used as a model to study the pathogenesis of AD and has been compared with reactions with haptens in allergic contact dermatitis (Kapsenberg *et al*, 1992; Werfel *et al*, 1997). In fact, in animal models of contact dermatitis it was found that epidermal cell-derived cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α play a major role in the sensitization and elicitation phase of this disease (Enk and Katz, 1992; Kondo *et al*, 1995). The application of haptens to the skin led to an upregulation of IL-1β and TNF-α as shown on protein and mRNA levels (Larsen *et al*, 1988; Boehm *et al*, 1996). Keratinocytes and Langerhans cells have been identified as cellular sources for these cytokines (Gueniche *et al*, 1994). They are important factors initiating inflammation as they induce adhesion molecules on endothelial cells and keratinocytes and are also able to activate Langerhans cells (Enk *et al*, 1993; Groves *et al*, 1995).

It may be hypothesized that, as observed with haptens in allergic contact dermatitis, protein allergens may be able to induce the synthesis of cytokines in keratinocytes and Langerhans cells in the skin of patients with AD. In this study, using the allergen patch test with HDM as a model, we tested the hypothesis that epidermis-derived TNF-α and IL-1β might also be involved in the pathogenesis of AD.

As opposed to hapten-induced patch test reactions with T helper 1 (Th 1) cells being the effector cells, in the allergen patch test with HDM, Th2 cells producing large amounts of IL-4 and little IFN-γ are thought to play an important role. This was shown with T cell clones and by polymerase chain reaction (PCR) analysis (Sager *et al*, 1992; Van Reijsen *et al*, 1992; Grewe *et al*, 1995; Neumann *et al*, 1996). We therefore asked the question whether IL-12, which has been shown to direct the differentiation of T cell subsets towards Th 1 cells (Trinchieri, 1995), shows an abnormal expression in the allergen patch test reaction, possibly contributing to the described dominance of Th 2 cytokines in AD skin.

Here we show that exposure to HDM leads to a significant upregulation of mRNA of IL-1β, TNF-α, and IL-12 p40 in the skin of sensitized patients with AD when compared with normal individuals. Increased levels of IL-12 p40 and TNF-α mRNA were only observed after the application of HDM, whereas IL-1β was also upregulated by the irritant sodium lauryl sulfate (SLS). Interestingly, epidermal control samples from healthy appearing skin of patients with AD exhibit significant levels of mRNA for IL-1β, TNF-α, and the subunit IL-12 p40 more often than epidermal cells of nonatopic individuals.

MATERIAL AND METHODS

Patients Sixteen adult patients with AD, classified according to the criteria established by Hanifin and Rajka (1980), were enrolled in this study. All patients

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Abbreviations: AD, atopic dermatitis; HDM, house dust mite antigen; SLS, sodium lauryl sulfate.

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had a positive prick test and specific IgE to HDM as detected by the carrier polymer system test (Pharmacia, Freiburg, Germany). Healthy individuals who served as controls (n = 8) had neither a history of atopy nor other skin diseases. This study was approved by the local ethics committee.

Patch tests At least 2 wk prior to testing, oral anti-inflammatory drugs were stopped and only emollients were allowed for treatment. The test site (back) had to be free of eczema for at least 1 mo. Patch tests were performed with 10 ug purified HDM (Bencard, Munich, Germany) dissolved in 20 μl saline. Patch tests with saline or SLS 3% (Sigma, Deisenhofen, Germany) served as controls. HDM patch tests were carried out in duplicate with Finn chambers (Hermal, Reinbeck, Germany). One test site was left untreated for further observation of the clinical patch test result. The other HDM-exposed skin site was scraped 8 h after the application of the allergen, as was the epidermis of SLS- or saline-exposed skin. A positive patch test, as defined by erythema with papules or vesicles, was observed at 48 h in six of 16 patients but in none of the healthy individuals serving as controls.

Skin sampling and RNA isolation Eight hours after the application of HDM, SLS, or saline as a negative control, epidermal cells were collected by scraping the epidermis off the patch test areas (1 cm^2) with a scalpel as described (Paludan and Thestrup-Pedersen, 1992). Bleeding was avoided, ensuring that only epidermal cells were collected. Cell samples were collected and stored in guanidinium thiocyanate solution (Sigma) at −70°C until further examination. Total RNA was extracted by a phenol-chloroform gradient and resuspended in 10 μl of distilled water.

Reverse transcriptase (RT)-PCR Reverse transcription was performed with 5 μl of each RNA probe at 42°C for 60 min, followed by 5 min at 96°C using random oligo primers pd (N)6 (Pharmacia) and 200 Units superscript reverse transcriptase (Gibco, Eggenstein, Germany). One tenth of the resulting cDNA was used per amplification. PCR was carried out with specific primers for:

IL-1β	sense, AAA CAG ATG AAG TGC TCC TTC CAG G anti-sense, TGG AGA ACA CCA CTT GTT GCT CCA product size, 388 bp
TNF-α	sense, GGC TCC ACC CTC TCT CCC CTG anti-sense, TCT CTC AGC TCC ACG CCA TTG product size, 394 bp
IL-12 p35	sense, GAG TCC CGG GAA AGT CCT GCC anti-sense, TCT GGC CTT CTG GAG CAT GTT product size, 313 bp
IL-12 p40	sense, GGG GTG ACG TGC GGA GCT GCT anti-sense, TCT TGC CCT GGA CCT GAA CGC product size, 343 bp
β-actin	sense, GAA ACT ACC TTC AAC TCC ATC anti-sense, CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG product size, 300 bp
IL-2	sense, ACT CAC CAG GAT GCT CAC AT anti-sense, AGG TAA TCC ATC TGT TCA GA product size, 259 bp
Interferon-γ	sense, AGT TAT ATC TTG GCT TTT CA anti-sense, ACC GAA TAA TTA GTC AGC TT product size, 355 bp
IL-4	sense, CTG CAA ATC GAC ACC TAT TAA anti-sense, CAG CTC GAA CAC TTT GAA TAT product size, 481 bp

All primers were purchased from MWG (Munich, Germany) or Biometra (Göttingen, Germany). PCR was performed on an Uno-Thermoblock (Biometra) using a 50 μl reaction mixture containing 2.5 U Taq DNA polymerase (Gibco), dNTP (each dNTP 0.2 mM, Pharmacia), $MgCl_2$ (1.5 mM), 125 pmol of sense and anti-sense primers, and 5 μl template cDNA in PCR-buffer (Gibco). The PCR consisted of 36 cycles of denaturation at 93°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 2 min. In the case of IL-12p35 a second PCR protocol with a hot start and 36 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 2 min was also performed. This protocol was used for the detection of IL-4 mRNA in part of the samples. PCR products were electrophoresed on 8% acrylamid gels and stained with ethidium bromide. Specificity of PCR products was controlled by comparing the localization of the bands with a DNA molecular weight standard (Boehringer, Mannheim, Germany) and diagnostic restriction enzyme digestion. PCR cycles without template cDNA were regularly performed for control, and also positive controls with cDNA obtained from phytohaemagglutinin-stimulated peripheral blood mononuclear cells were run in parallel.

Quantitation of PCR products Gels were stained with ethidium bromide, scanned with a video-based densitometer, and analyzed with the Scan Pack II software (Biometra). Densitometric analysis of PCR products included tails

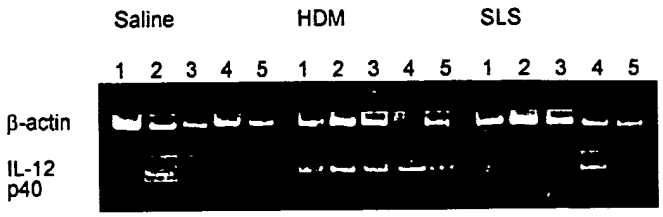

Figure 1. β-actin and IL-12 p40 mRNA expression in uninvolved epidermis of patients with AD. IL-12 p40 and β-actin PCR products of five representative patients are shown. Although β-actin was detectable in each sample, IL12 p40 mRNA was almost exclusively expressed in HDM-exposed epidermis.

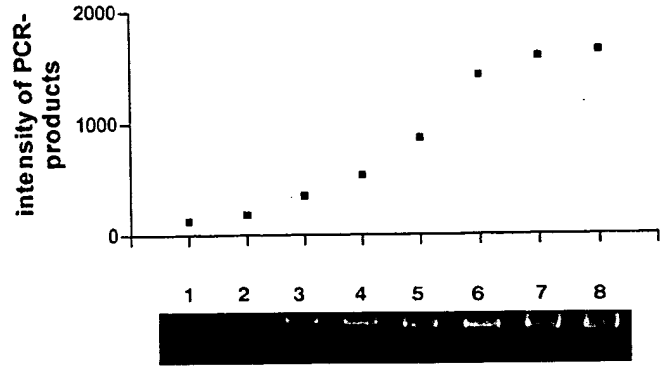

Figure 2. PCR for β-actin with different amounts of cDNA. cDNA dilutions were as follows: *lane 1*, 1:128; *lane 2*, 1:64; *lane 3*, 1:32; *lane 4*, 1:16; *lane 5*, 1:8; *lane 6*, 1:4; *lane 7*, 1:2; *lane 8*, 1:1. The signal strength of the PCR products increase with increasing amounts of cDNA.

when present on the gels. The intensities of bands formed by the cytokine PCR products of interest were put into relation to the intensity of the band derived from the β-actin-PCR product of the same sample. So, the resulting ratios for IL-12 p40, TNF-α, and IL-1β were normalized to β-actin mRNA, which is constitutively expressed. Upregulation of cytokine mRNA in the epidermis was defined either as newly detected mRNA or as a significant enhancement of the intensity of PCR bands of cytokines normalized to β-actin bands. Statistical analysis was performed using the Wilcoxon signed rank test or the Mann–Whitney test calculated by commercial software (Prism 2.01, Graph Pad, San Diego, CA).

RESULTS

Standardization of PCR A PCR protocol with 36 cycles was chosen in order to ensure maximal sensitivity. All epidermal samples in this study were positive for β-actin mRNA; however, β-actin signals varied between samples (**Fig 1**) It therefore was important to ensure that the signals obtained were suitable for quantitation. In **Fig 2** we show that the densitographic intensity of PCR products for β-actin is a function of the amount of cDNA applied to the reaction mixture. This was also confirmed for all cytokines (data not shown).

Epidermal cytokines in healthy individuals TNF-α mRNA was rarely detectable in epidermal samples of healthy individuals independently from the agents applied to the skin (**Fig 3*a***). Messenger RNA of the housekeeping gene β-actin, however, was readily detectable in all samples derived from healthy individuals (data not shown). When compared with β-actin, exposure to HDM led to an upregulation of TNF-α in only two of the healthy individuals with a negative saline sample. Furthermore, mRNA for IL-1β was negative in seven of eight epidermal samples obtained from healthy individuals and this did not differ in skin that had been exposed to HDM, saline, or SLS (**Fig 4*a***). Only one individual showed high levels of IL-1β after the application of saline and HDM as well. This individual on the other hand showed an upregulation of neither TNF-α nor IL-12 p40.

Also, IL-12 p40 mRNA was detectable in the epidermis of one individual only and was only slightly upregulated in two of eight individuals after exposure to HDM and SLS (**Fig 5*a***). Interestingly, these were the same individuals who also showed upregulation of

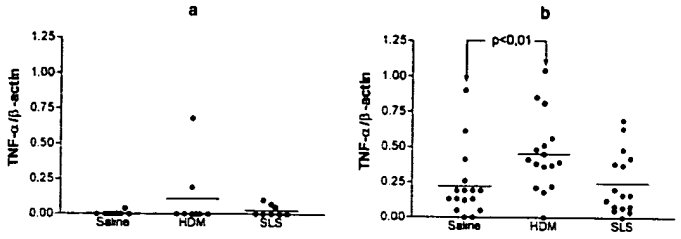

Figure 3. TNF-α mRNA expression in the epidermis 8 h following patch tests with saline, HDM, and SDS. Optical densities of RT-PCR products normalized to β-actin are shown (ratio of TNF-α mRNA and β-actin mRNA). p values were calculated using the Wilcoxon test. (*a*) Epidermal samples obtained from healthy individuals; (*b*) epidermal samples obtained from patients with AD.

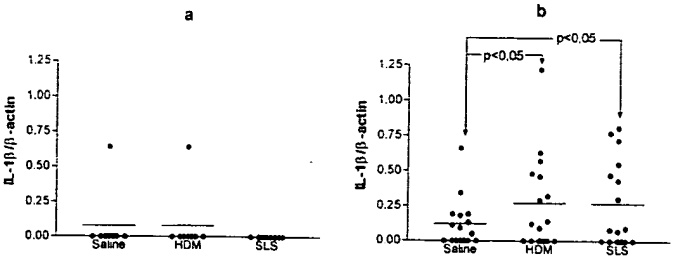

Figure 4. IL1-β mRNA expression in the epidermis of healthy individuals 8 h following patch tests with saline, HDM, or SDS. Optical densities of RT-PCR products normalized to β-actin are shown (ratio of IL-1β mRNA and β-actin mRNA). p values were calculated using the Wilcoxon test. (*a*) Epidermal samples obtained from healthy individuals; (*b*) epidermal samples obtained from patients with AD.

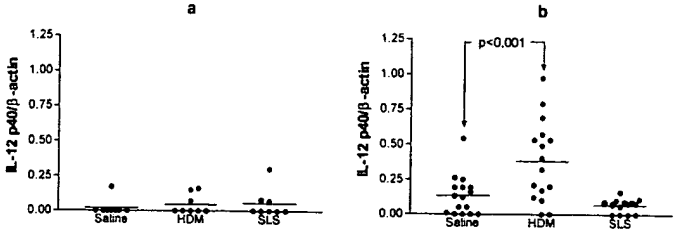

Figure 5. IL-12 p40 mRNA expression in the epidermis of healthy individuals 8 h following patch tests with saline, HDM, or SDS. Optical densities of RT-PCR products normalized to β-actin are shown (ratio of IL-12 p40 mRNA and β-actin mRNA). p values were calculated using the Wilcoxon test. (*a*) Epidermal samples obtained from healthy individuals; (*b*) epidermal samples obtained from patients with AD.

TNF-α mRNA after the application of HDM and SLS to their skin, pointing to an allergen-independent irritation.

Epidermal cytokines in patients with AD In contrast to nonatopic healthy individuals, in patients with AD TNF-α mRNA was detectable in 14 of 16 of epidermal samples derived from saline-exposed skin (**Fig 3*b***). Also, IL-1β mRNA was detected in nine (**Fig 4*b***) and IL-12 p40 mRNA in 11 of 16 patients (**Fig 5*b***).

As further shown in **Figs 3–5**, HDM led to a significant further upregulation of TNF-α mRNA when compared with saline. Normalized to β-actin mRNA, 75% of patients showed enhanced levels ($p < 0.01$). Although there was a wide range of responses, IL-1β was also significantly enhanced ($p < 0.05$) by HDM. Six patients stayed negative with either agent tested. Strongest upregulation by HDM was observed for IL-12 p40 mRNA.

SLS induced TNF-α mRNA in seven of 16 patients (44%), but this did not differ significantly from the results obtained with saline-exposed skin. In contrast to TNF-α, SLS significantly increased IL-1β mRNA above saline levels ($p < 0.05$). As observed with HDM, SLS was also not able to induce IL-1β in six of 16 patients. As observed with TNF-α and IL-1β, HDM patch testing led to a strong further enhancement of IL-12 p40 mRNA ($p < 0.001$); however, different from the results

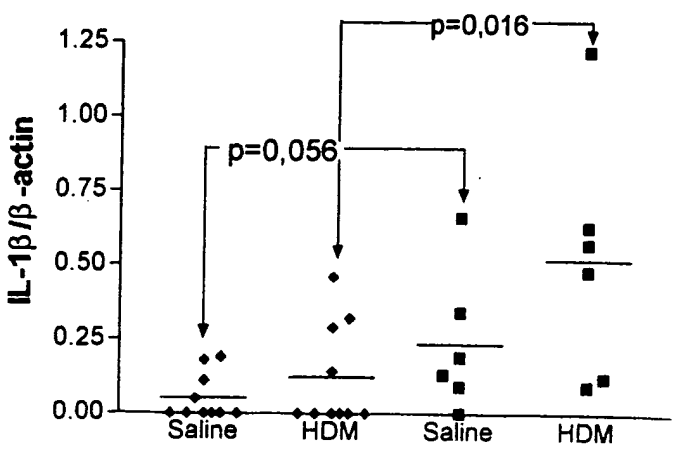

Figure 6. IL-1β mRNA expression in the epidermis of patch-test positive and negative patients with AD. Eight hours after HDM application significantly higher levels of IL-1β mRNA are found in patch positive patients compared with patch negative patients. Optical densities of RT-PCR products normalized to β-actin are shown (ratio of IL-1β mRNA and β-actin mRNA). For statistical analysis the Mann–Whitney test was used. ◆, Patch negative patients; ■, patch positive patients.

obtained for IL-1β, upregulation of IL-12 p40 mRNA was specific for HDM as the application of SLS yielded only background levels. Selected cases are illustrated in **Fig 1**, showing a blot with the PCR products of β-actin and IL-12 p40 obtained with epidermal samples derived from five patients. Although β-actin was detectable in each sample, IL-12 p40 mRNA was not found in most samples derived from saline- or SLS-exposed skin. IL-12 p35 mRNA was not detectable either in any of the epidermal samples derived from the skin of AD patients or in healthy controls when the PCR protocol with an annealing temperature of 60°C was used. When PCR was performed with a hot start and an annealing temperature of 52°C, a faint band could be detected in two of seven epidermal samples of HDM patch tests; however, in these cases the signals were too faint to be suitable for densitographic analysis. Controls with phytohaemagglutinin-stimulated peripheral blood mononuclear cells were routinely positive for IL-12 p35 mRNA regardless of the different PCR protocols used (data not shown).

Together these results point to an upregulation of certain epidermal cytokines in untreated healthy appearing skin of patients with AD when compared with healthy skin of normal individuals. Moreover, the application of HDM led to a further upregulation of TNF-α and IL-12 p40 mRNA in 75% and of IL-1β in ≈50% of these patients, indicating a characteristic reaction pattern in the patients' group only.

Comparison of epidermal cytokines in patch test positive and negative patients with AD The results described so far do not discriminate between individual patients who, as proved by parallel testing, reacted positive or negative to HDM. As there were considerable variations in cytokine mRNA levels among individual AD patients, we asked whether quantitative levels of cytokine mRNA might be related to the clinical outcome of the HDM patch tests. In fact, **Fig 6** shows that the upregulation of IL-1β after the application of HDM was more pronounced in those individuals who subsequently developed a positive patch test ($p = 0.016$). Moreover, although not significant, five of six patients with positive patch tests showed IL-1β mRNA in saline control samples compared with only four of 10 patch test negative atopic individuals. It therefore may be speculated that constitutive levels of certain cytokines in the healthy appearing skin of AD patients might govern the individual outcome of the HDM patch tests. Although enhanced basic levels of IL-1β mRNA in healthy appearing skin may help to identify those individuals who develop a positive HDM patch test, neither quantitation of TNF-α mRNA nor IL-12 p40 mRNA aided in predicting the clinical outcome of the patch test (data not shown).

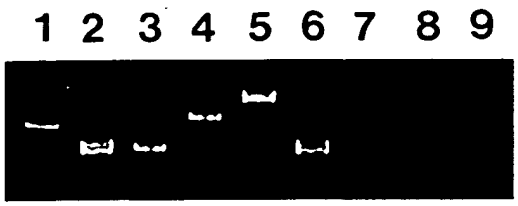

Figure 7. PCR for T cell derived cytokines. PCR for β-actin (*lanes 2* and *6*), IL-2 (*lanes 3* and *7*), IFN-γ (*lanes 4* and *8*), and IL-4 (*lanes 5* and *9*) was performed. In *lane 1* a DNA marker with a prominent band at 350 bp is shown. When cDNA of phytohaemagglutinin-stimulated peripheral blood mononuclear cells was used (*lanes 2–5*) mRNA for all cytokines could be detected. An epidermal sample of a HDM patch test (8 h) of an atopic individual is positive for β-actin (*lane 6*) and negative for IL-2 (*lane 7*), IFN-γ (*lane 8*), and IL-4 (*lane 9*). A representative experiment out of seven is shown.

DISCUSSION

This study shows that upregulation of the epidermal cytokines TNF-α, IL-1β, and IL-12 p40 8 h after the application of protein antigens of the house dust mite to the skin is a frequent event in the epidermis of AD patients who are sensitized to these antigens but not in the epidermis of normal individuals. Also, control samples from patients with AD showed enhanced cytokine mRNA. As immunohistologic studies have shown that the number of T cells in the epidermis of saline-exposed, healthy appearing skin of AD patients is low (Jung *et al*, 1996), these cytokines are most probably produced by Langerhans cells and or keratinocytes. To confirm that epidermal T cells had not contributed to the observed levels of IL-1β and TNF-α, we also performed RT-PCR for T cell derived cytokines in selected epidermal samples. We were not able to detect mRNA for IFN-γ, IL-2, or IL-4 in these samples, excluding the possibility that the observed increase in cytokine mRNA was attributable to epidermal T cells (**Fig 7**).

It is recognized that the PCR method used in this study previously has been reported to yield variable results (Sambrook *et al*, 1989). In this study, in order to achieve standardization of the method, equivalent amounts of RNA samples were used for cDNA synthesis. Standardization was satisfying as background cytokine levels within each group (healthy and atopic individuals) showed little interindividual differences, allowing a semiquantitative estimation of PCR products for the cytokines investigated.

As expected, in saline-exposed epidermis of healthy individuals, significant mRNA for the cytokines of interest were not detectable with the exception of 2–3 cases. In contrast, the majority of AD patients showed considerable levels of TNF-α, IL-1β, or IL-12 p40 in saline-exposed epidermis. This was surprising, because the patients had been free of skin disease for at least 4 wk prior to sampling. These results point to a constitutive upregulation of certain cytokines in the epidermis of most AD patients. Both keratinocytes and Langerhans cells are known to produce TNF-α, IL-1β, and IL-12. It was shown that Langerhans cells seem to be the main epidermal source for IL-12 p40 (Heufler *et al*, 1996) and IL-1β, whereas TNF-α is produced in considerable amounts by keratinocytes (Enk and Katz, 1992). As human Langerhans cells were also reported to produce TNF-α (Larrick *et al*, 1989), both types of cells could have contributed to the observed increase of TNF-α mRNA. It has been shown that an impaired barrier function of the skin leads to activation of Langerhans cells (Proksch *et al*, 1996), and impaired barrier function of the skin is one of the characteristic features of AD (Werner and Lindberg, 1985). It recently was reported that a G to A transitional polymorphism at position −308 of the promoter region of the TNF-α gene can be correlated positively with increased skin irritability.[2] Whether such polymorphisms may also be responsible for the increased levels of TNF-α mRNA in nonlesional AD skin remains to be clarified. Clearly, increased cytokine mRNA in healthy appearing skin of patients with AD may help to explain the increased skin irritability observed in these individuals as TNF-α and IL-1β, which are known to induce adhesion molecules and cell migration by acting on the dermal microvasculature, may facilitate the recruitment of inflammatory cells to the skin (Leung *et al*, 1991). This hypothesis is also supported by our recent observation that VCAM-1 is upregulated on dermal vessels in healthy appearing skin of patients with AD (Jung *et al*, 1996). Moreover, the same mechanisms may facilitate contact sensitization to HDM as evidenced by the development of a positive allergen patch test.

The data presented in this paper allow the conclusion that, analogous to previous reports with haptens in individuals without atopy, in the skin of sensitized atopic individuals protein allergens are able to induce a strong activation of those epidermal cytokines that are essential for the development of a delayed type reaction. This observation supports the present concept of AD being a T cell mediated reaction of the skin in which trapping of protein allergens, mediated by IgE receptors on Langerhans cells, may be an important disease mechanism (Bieber, 1997). The allergen patch test in AD patients is regarded as a model for an antigen-specific response of the skin to protein allergens, as the application of HDM is followed by a characteristic delayed type hypersensitivity reaction harbouring significant numbers of allergen specific T cells (Ramb-Lindhauer *et al*, 1991; Sager *et al*, 1992; Van Reijsen *et al*, 1992). SLS on the other hand, is known to provoke an irritant dermatitis that clinically shows a decrescendo reaction pattern (Lee *et al*, 1997). Interestingly, as opposed to reports with haptens in contact allergic nonatopic individuals, we show that SLS induced a similar upregulation of IL-1β in atopic skin as observed with HDM. This may result from a stronger penetration of SLS as a consequence of the impaired barrier function of healthy appearing atopic skin. TNF-α on the other hand, and even more so IL-12 p40, showed a significantly stronger upregulation after the application of HDM than SLS. Thus, a strong upregulation of these latter two cytokines in the skin seems to be characteristic for the exposure to allergens.

As human Langerhans cells have been reported to synthesize more IL-12 than keratinocytes (Kang *et al*, 1996), the observed increase of IL-12 p40 mRNA after the application of HDM to the skin of sensitized AD patients appears to be attributable to specific activation of Langerhans cells. In fact, IL-12 p40 showed the strongest upregulation of mRNA levels of all cytokines investigated. The function of IL-12 p40 in HDM-exposed skin is not clear. It is well known that the complete IL-12 molecule, comprised of IL-12 p40 and IL-12 p35, is able to strongly enhance the Th 1 pathway by inducing IFN-γ production (Germann *et al*, 1993). With the methods employed we were not able to detect sufficient amounts of IL-12 p35 mRNA for densitometric analysis. Previous reports on this issue have shown that keratinocytes stimulated with phorbol dibutyrate are able to express IL-12 p35 mRNA (Aragane *et al*, 1994). Constitutive expression of IL-12 p35 mRNA in keratinocytes was only detectable with very sensitive techniques such as radioactive hybridization of PCR products or nested PCR (Müller *et al*, 1994; Yawalkar *et al*, 1996). In enriched Langerhans cell samples IL-12 p35 could be detected by PCR (Heufler *et al*, 1996). In agreement with our own results other authors have shown that IL-12 p40 is usually expressed in considerable excess to IL-12 p35 (Trinchieri, 1995).

The IL-12 p40 protein, however, when present as homodimer, has been described to antagonize the promoting effect on Th 1 cells exerted by the complete IL-12 molecule (Gillessen *et al*, 1995; Ling *et al*, 1995). It therefore might be speculated that the observed induction of high levels of IL-12 p40 mRNA early after the application of HDM to atopic skin may be causally involved in the development of the Th-2 dominated response early in the atopy patch test reaction. This suggests the possibility that IL-12 p35, as found by Grewe *et al* 24 h after HDM application, as well as a corresponding Th 1 cytokine pattern, may play a major role later in the allergen patch test reaction (Grewe *et al*, 1994, 1995). Further investigations, comparing IL-12 p40 mRNA levels after the application of Th 1-inducing haptens to the skin, may help to answer this question.

The group of AD patients, although sensitized to HDM, is known to be heterogeneous with respect to their capacity to develop a positive reaction to HDM (Darsow *et al*, 1995). Although all three cytokines

[2]Wakelin SH, Allen MH, Holloway D, Baadsgard O, Barker JN, McFadden J: TNFα promoter region polymorphisms and irritant susceptibility. *J Invest Dermatol* 109:412, 1997 (abstr.)

were upregulated in the majority of our patients after HDM had been applied to the skin, this upregulation was followed by a clinically visible reaction in only six of 16 patients. This rate is within the range previously observed by others (Van Voorst Vader *et al*, 1991) and shows that upregulation of these cytokines per se was not sufficient to develop a positive patch test: however, the skin of patch test positive individuals was characterized by a stronger upregulation of IL-1β than the skin of negative individuals ($p = 0.016$). This indicates that a strong upregulation of IL-1β is essential for the development of eczema after skin contact with HDM in sensitized AD patients. Neither the increase of TNF-α mRNA nor the increase of IL-12 p40 mRNA showed a correlation with the clinical outcome (results not shown). Thus, although the upregulation of IL-12 p40 suggests allergen specificity, the activation of this cytokine may be essential but not sufficient for the development of eczema in atopic patients.

Finally, individuals who, prior to the application of HDM, exhibited a high level of IL-1β mRNA in their epidermis, appeared to be more prone to developing a clinical response as evidenced by a positive patch test (**Fig 5**). This finding was restricted to IL-1β. Although not reaching significance with the number of individuals investigated, this observation may be of special importance. It seems to indicate also that the basic level of IL-1β in normal appearing skin in individuals with AD, possibly reflecting a certain activation state of Langerhans cells, may help to predict whether eczema will be provocable by HDM. Thus, future therapeutic considerations may include the reduction of basic IL-1β levels in order to avoid exacerbation of AD by exogenous contact with protein allergens.

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Cytokine mRNA expression in human epidermis after patch treatment with rhus and sodium lauryl sulfate.

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BACKGROUND: Cytokines have been shown to play a pivotal role in the development and elicitation of contact hypersensitivity reactions. The sources of these cytokines in the skin include T cells, keratinocytes, and Langerhans cells. OBJECTIVE: In an effort to characterize the cytokines involved in the elicitation phase of a contact allergic response, we examined mRNA expression in human epidermis following patch testing with a known allergen and vehicle. METHODS: Allergic subjects were patch tested with poison ivy allergen (rhus), irritant (sodium lauryl sulfate [SLS]) and vehicle controls for 24 hours. Epidermal samples were obtained from the patch sites by a suction blister technique. Total RNA was isolated from the epidermis and the level of cytokine gene expression was determined using reverse transcriptase polymerase chain reaction (RT-PCR). PCR products for the various cytokines were confirmed and semiquantitated by liquid hybridization with (32)P-labeled product-specific probes. RESULTS: Results of liquid hybridization confirmed the presence of message for interleukin (IL)-2, IL-4 and IL-10 in rhus, SLS, and vehicle treated sites. Generally, in rhus treated sites, the steady state level of message for IL-2 was highest, followed by IL-4 and IL-10, in decreasing levels. In contrast, only minimal expression of mRNA for these cytokines was observed in irritant and vehicle treated sites. Interestingly, interferon (IFN)-gamma mRNA was not detected at 24 hours in rhus, SLS, or vehicle treated sites. CONCLUSION: These preliminary results indicate differences in the steady state levels of cytokine mRNA in allergen versus vehicle and irritant treated sites at 24 hours after treatment.

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Pathogenesis of Drug-Induced Exanthema

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Key Words

T cells · Perforin · Granzyme B · IL-5 · Drug allergy · Immunohistochemistry

Abstract

Background: In vitro data derived from drug-specific T cell clones have revealed that heterogeneous T cell subsets with distinct phenotypes (CD4+ > CD8+) and cell functions (strong IL-5 production, cytotoxic potential) are generated. The aim of this study was to elaborate the relevance of these findings in vivo. ***Methods:*** Skin biopsy specimens from drug-induced maculopapular exanthema and normal skin were analyzed for CD4, CD8, CD25, HLA-DR, CD54, perforin, granzyme B, IL-5 and IFN-γ using immunohistochemistry. ***Results:*** The majority of infiltrating lymphocytes in maculopapular drug eruptions were CD4+. Both CD4+ and CD8+ T cells expressed perforin and granzyme B and were partly located at the dermoepidermal junction and in the epidermis. In addition, strong immunoreactivity for IL-5 and moderate immunoreactivity for IFN-γ were observed in the mononuclear cell infiltrate. ***Conclusions:*** Our data indicate that skin infiltrating T cells with a cytotoxic potential and the ability to produce IL-5 and IFN-γ may contribute to the damage of keratinocytes and the activation of eosinophils, which are typical features of drug-induced maculopapular exanthema.

Introduction

In recent years increasing evidence has indicated an important role for drug-specific T cell in drug allergy [1]. Previous reports have revealed that chemically reactive drugs like β-lactam antibiotics are able to directly modify proteins and peptides and change the MHC-embedded peptides in a covalent way [2, 3]. This hapten modification leads to the generation of new immunogenic determinants and thus enables immunogenicity of an autologous peptide. In addition to this hapten model, our group has demonstrated that chemically inert drugs like lidocaine or sulfamethoxazole (SMX) do not require processing to a reactive metabolite before binding to proteins or peptides, since glutaraldehyde-fixed antigen-presenting cells (APC) can present SMX or lidocaine to T cell clones (TCC) [4]. These results suggest that T cells are able to recognize certain drugs directly, which might explain allergic reactions to drugs in organs without the ability of drug metabolism. Furthermore, analysis of drug-specific TCC revealed that heterogeneous T cell subsets with distinct phenotypes and cell functions are generated. The majority of our drug-specific TCC had a CD4+ phenotype and produced increased amounts of IL-5 [5]. As a particular interesting finding SMX-specific TCC of both CD4+ and CD8+ phenotypes showed a drug-specific and MHC-restricted cytotoxicity against autologous B lymphoblasts [4]. In addition, autologous keratinocytes were also specifically killed predominantly by CD4+ TCC [6]. These in vitro findings indicate

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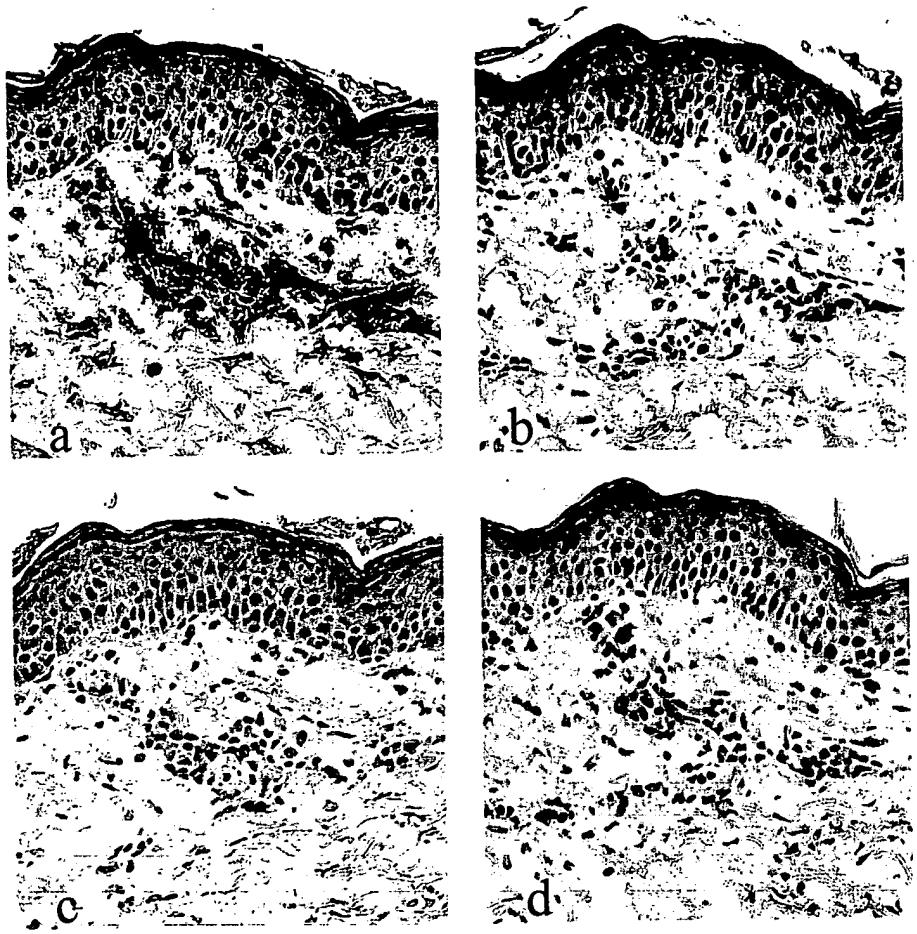

Fig. 1. Expression of CD4 (**a**), CD8 (**b**), perforin (**c**) and granzyme B (**d**) in drug-induced maculopapular exanthema. Original magnification × 250.

that cytotoxicity mediated by drug-specific T cells may represent an important pathway in certain forms of cutaneous adverse drug reactions. To address the question of how these in vitro elaborated mechanism apply to the in vivo situation, we performed the following study.

Materials and Methods

Nine Caucasian patients with a typical, acute, generalized, maculopapular drug eruption were included in the study after obtaining their informed consent. The culprit drug was identified in all patients by a clear history, positive scratch-patch and/or in vitro tests (lymphocyte transformation tests). Control biopsies were obtained from patients undergoing reconstructive surgery.

Punch biopsy specimens were equally divided into two pieces. For standard histology one half was fixed in 4% formalin, routinely processed, paraffin-embedded and stained with hematoxylin and eosin. The second half of the skin biopsy specimen was used for immunohistochemical analysis of granzyme B (Hölzel Diagnostik, Köln, Germany), IL-5 (Pharmingen/Becton Dickinson, Mountain View, Calif., USA) and IFN-γ (Ancell, Bayport, Minn., USA) using the alkaline phosphatase anti-alkaline phosphatase method [7]. Immunostaining for perforin (Ancell), CD4, CD8, CD25, HLA-DR and CD54 (all obtained from DAKO, Glostrup, Denmark) was performed using the avidin-biotin complex/alkaline phosphatase method [7]. Double immunostaining was performed for the cytotoxic molecules and CD4 or CD8 [7]. Substitution of the primary antibody with an irrelevant isotype-matched IgG and omission of the primary antibody served as negative controls.

Results

The skin sections showed a superficial, mixed inflammatory cell infiltration, which was predominantly composed of lymphocytic cells. Eosinophils were found in 7 of the 9 cases. Furthermore, focal interface changes with vacuolar alteration in the basal cell layer, some scattered individual dyskeratotic and necrotic keratinocytes were observed. Immunohistochemistry revealed that the great majority of infiltrating lymphocytes in maculopapular drug eruptions were CD4+ T cells (fig. 1a). Interestingly,

the majority of the basal keratinocytes stained strongly for HLA-DR and focally for CD54 (intercellular adhesion molecule-1, ICAM-1), indicating that keratinocytes are activated in these drug eruptions and may also act like APC and present antigens to CD4+ T cells. In contrast to normal skin, a pronounced enhancement of perforin and granzyme B expression was observed in drug-induced cutaneous lesions (fig. 1c, d). Double staining for cytotoxic molecules and CD4 or CD8 indicated that both cell types express perforin and granzyme B. Furthermore, immunoreactivity was strongly increased for IL-5 and moderately for IFN-γ in the maculopapular drug eruptions and mainly located within the inflammatory cell infiltrate.

Discussion

Our data indicate that skin-infiltrating T cells with a cytotoxic potential and the ability to produce IL-15 and IFN-γ contribute to tissue damage and the activation of eosinophils in drug-induced, maculopapular exanthema. By extending previous in vitro data obtained with T cells from the peripheral blood with these ex vivo immunohistochemical studies, we propose the following concept as the pathomechanism of drug-induced maculopapular exanthema. Both chemically reactive and nonreactive drugs are presented by APC in the dermis/epidermis and eventually by other cells, i.e. activated keratinocytes. The chemically reactive drugs bind covalently, whereas the nonreactive drugs do not need metabolism to be presented and can bind from the outside in a noncovalent way to MHC class I and II. In the event that sufficient drug-specific T cells are activated, various cytokines (e.g. IL-5, IFN-γ) and chemokines will be secreted by these cells. This would lead to the upregulation of MHC II and adhesion molecules on resident cells, i.e. endothelial cells and keratinocytes as well as the further stimulation of cytokine and chemokine production by these cells. The infiltrating T cells will be composed of CD4 and CD8 cells, whereby the ratio of this composition may determine the type of exanthema as cytotoxic, CD4+ T cells infiltrating into the epidermis may kill MHC class II- and ICAM-1-expressing keratinocytes in a drug-specific way and be *mainly* involved in eliciting the maculopapular exanthema. The vacuolar alteration in the basal cell layer with some necrotic keratinocytes would represent the histological correlate of this T cell-mediated cytotoxicity. Upregulation of cytokines like IL-5 and chemokines like eotaxin may recruit eosinophils, which further amplify the underlying immune response. Taken together, this concept might also explain the enhancing role of viral infections on drug allergy, namely via increased IFN-γ production and upregulation of MHC class II on keratinocytes, which enables drug presentation.

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